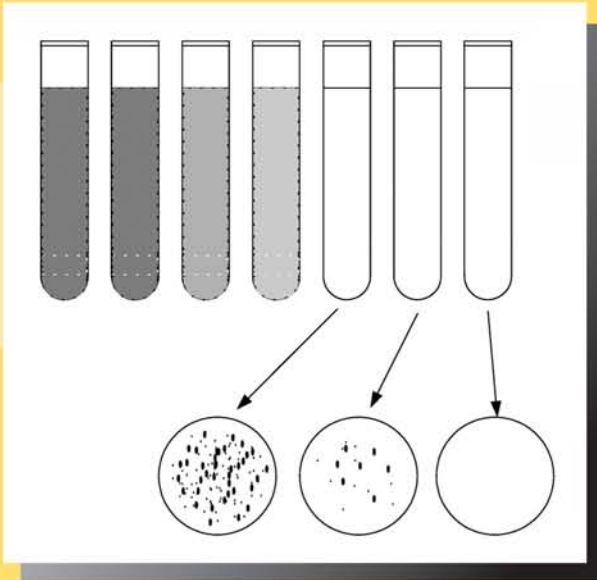
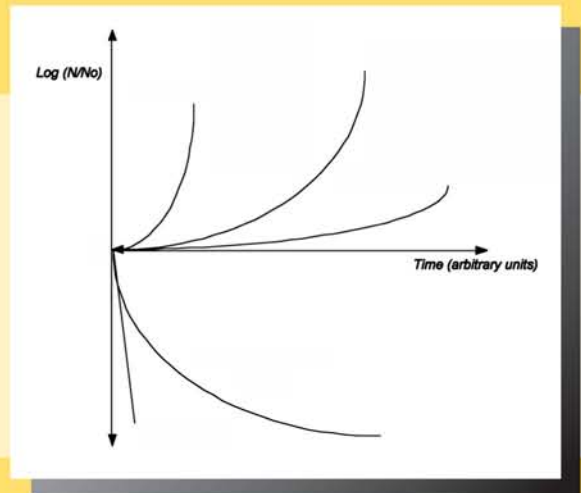


ANTIMICROBIALS IN FOOD



*Third
Edition*

Edited by
P. Michael Davidson
John N. Sofos
A. L. Branen



**ANTIMICROBIALS
IN FOOD**

Third Edition

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ANTIMICROBIALS IN FOOD

Third Edition

Edited by

P. Michael Davidson

John N. Sofos

A. L. Branen



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Preface

The consequences of unintended microbial growth in foods are hazards due to the presence of pathogenic microorganisms or economic losses due to spoilage microorganisms. Preservation technologies are designed to protect foods from the effects of microorganisms and inherent deterioration. Microorganisms in foods may be inhibited or inactivated by physical methods (e.g., heat, cold, reduced water activity) or through application of antimicrobial compounds. Food antimicrobials, including chemical sanitizers, may be broadly defined as chemical compounds present in or added to foods, food packaging, food contact surfaces, or food processing environments that inhibit the growth of, or inactivate, pathogenic or spoilage microorganisms. Historically, the primary function of food antimicrobials has been to prolong shelf life and preserve quality through the inhibition of spoilage microorganisms. In the past 10 to 15 years, however, antimicrobials have been increasingly utilized as a primary intervention for the inhibition or inactivation of pathogenic microorganisms in foods. This function becomes increasingly important as food processors search for more and better tools to improve food safety.

Antimicrobials continue to be one of the most important classes of food additives. Research on antimicrobials, especially naturally occurring compounds, has increased dramatically in the past 10 to 15 years. The primary incentive for searching for effective antimicrobials among naturally occurring compounds is to expand the spectrum of antimicrobial activity over that of the traditional, regulatory-approved substances. Most of the traditional food antimicrobials have limited application due to pH or food component interactions. Interest in natural antimicrobials is also driven by the fact that international regulatory agencies are generally very strict as to requirements for toxicological evaluation of novel direct food antimicrobials. An argument often used to justify natural antimicrobials is that they will produce “green” labels (i.e., with few or no “synthetic” additives in the ingredient list). However, this justification may lead consumers to the mistaken belief that antimicrobial food additives currently in use are potentially toxic and should be avoided. In short, natural antimicrobials have excellent potential but likely will not produce miracles. This has not stopped researchers from continuing to look for the “perfect” food antimicrobial. However, a single compound that is effective against all microorganisms in all storage situations and in all foods likely does not exist. More research is needed on the effectiveness of antimicrobial combinations and antimicrobials in combination with physical methods (e.g., hurdle technology) that are effective against different groups of microorganisms. Combinations could well be the ideal antimicrobial for which everyone is searching.

It has been approximately 12 years since the second edition of *Antimicrobials in Foods* was published. In that time, many changes have taken place in the field of food microbiology and the research area of food antimicrobials. At the time of the second edition, major outbreaks of *Escherichia coli* O157:H7 and *Listeria monocytogenes* had not occurred, consumer and regulatory demands for improved food safety were only beginning, and use of naturally occurring antimicrobials was in its infancy. At the time of the second edition, lysozyme, lactoferrin, ozone, and several other compounds were not approved for use in or on foods in the United States. Since the time of the second edition, a great deal of progress has been made on determining the spectrum of action, environmental effects on activity, and mechanisms of action of a number of naturally occurring antimicrobials.

Because of the many changes since the second edition of *Antimicrobials in Foods*, considerable revisions have been made for this third edition. As previously, one thing that has not changed is the excellent international reputations of the authors of each chapter. Some of the authors are new,

but, as in the second edition, all are well-recognized experts on their topics. The format for each chapter varies somewhat depending on the areas each author wishes to emphasize. In general, chapters on specific antimicrobials include information on spectrum of activity, application to foods, mechanisms or potential mechanisms of action, regulations, toxicological aspects, and assay in foods. In the third edition, five new chapters have been added. New chapters on specific antimicrobials include those on lysozymes, naturally occurring antimicrobials from animal sources, and naturally occurring antimicrobials from plant sources. In addition, chapters have been added on hurdle technology and mechanisms of action, resistance, and stress adaptation. All existing chapters were extensively revised to reflect research that has taken place since 1993, the publication date of the second edition. The editors would like to thank the chapter authors for their dedication and hard work in producing their excellent works. We sincerely hope that these discussions will continue to be useful for industrial, academic, and regulatory research scientists, technical advisors, industry consultants, regulators, consumers, and consumer advocates regarding the potential value of antimicrobials in the food supply.

P. Michael Davidson

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Editors

P. Michael Davidson is a professor of food microbiology at the University of Tennessee, Knoxville. Serving on the editorial board of several journals and as scientific coeditor of the *Journal of Food Protection*, Dr. Davidson is the author or coauthor of more than 150 journal articles, book chapters, and abstracts. He is a Fellow of the American Academy of Microbiology and the Institute of Food Technologists (IFT) and is a member of the American Society for Microbiology (ASM), the Society for Applied Microbiology, and the International Association of Food Protection. He served as chair of the food microbiology divisions of both the ASM and IFT. Dr. Davidson received the B.S. degree (1972) from the University of Idaho, Moscow, the M.S. degree (1977) from the University of Minnesota, Minneapolis-St. Paul, and the Ph.D. degree (1979) in food science and technology from Washington State University, Pullman.

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Contents

Chapter 1

Food Antimicrobials — An Introduction.....1
P. Michael Davidson and A.L. Branen

Chapter 2

Sodium Benzoate and Benzoic Acid11
John R. Chipley

Chapter 3

Sorbic Acid and Sorbates.....49
Jarret D. Stopforth, John N. Sofos, and Francis F. Busta

Chapter 4

Organic Acids.....91
Stephanie Doores

Chapter 5

Sulfur Dioxide and Sulfites143
Cornelius S. Ough and Lilian Were

Chapter 6

Nitrite169
R. Bruce Tompkin

Chapter 7

Nisin237
Linda V. Thomas and Joss Delves-Broughton

Chapter 8

Natamycin275
Joss Delves-Broughton, Linda V. Thomas, Craig H. Doan, and P. Michael Davidson

Chapter 9

Parabens.....291
P. Michael Davidson

Chapter 10

Dimethyl Dicarboxylate and Diethyl Dicarboxylate305
David A. Golden, Randy W. Worobo, and Cornelius S. Ough

Chapter 11	
Medium-Chain Fatty Acids and Esters.....	327
<i>Jon J. Kabara and Douglas L. Marshall</i>	
Chapter 12	
Lysozyme	361
<i>Eric A. Johnson and Ann E. Larson</i>	
Chapter 13	
Bacteriocins with Potential for Use in Foods	389
<i>Dallas G. Hoover and Haiqiang Chen</i>	
Chapter 14	
Naturally Occurring Compounds — Plant Sources	429
<i>Aurelio López-Malo Vigil, Enrique Palou, and Stella M. Alzamora</i>	
Chapter 15	
Naturally Occuring Compounds — Animal Sources	453
<i>Jarret D. Stopforth, Panos N. Skandamis, P. Michael Davidson, and John N. Sofos</i>	
Chapter 16	
Sanitizers: Halogens, Surface-Active Agents, and Peroxides	507
<i>Bruce R. Cords, Scott L. Burnett, John Hilgren, Matthew Finley, and Joshua Magnuson</i>	
Chapter 17	
Indirect and Miscellaneous Antimicrobials	573
<i>Leora A. Shelef and Julie Seiter</i>	
Chapter 18	
Antibiotic Residues in Foods and Their Significance.....	599
<i>Stanley E. Katz and Paula Marie L. Ward</i>	
Chapter 19	
Update on Hurdle Technology Approaches to Food Preservation.....	621
<i>Lothar Leistner and Grahame W. Gould</i>	
Chapter 20	
Mechanisms of Action, Resistance, and Stress Adaptation	633
<i>A.D. Russell</i>	
Chapter 21	
Methods for Activity Assay and Evaluation of Results	659
<i>Aurelio López-Malo Vigil, Enrique Palou, Mickey E. Parish, and P. Michael Davidson</i>	
Index	681

1 Food Antimicrobials – An Introduction

P. Michael Davidson and A.L. Branen

CONTENTS

Role of Additives in Food.....	1
Definition and Function of Chemical Food Preservatives	2
Selection of Antimicrobials	3
Antimicrobial Spectrum.....	4
Physicochemical Properties of the Antimicrobial	5
Food-Related Factors	5
Process Factors.....	6
Considerations in the Use of Food Antimicrobials	7
Sanitation	7
Toxicologic Safety	7
Labeling	8
Economics of Use.....	8
Activity Validation Methods.....	8
Sensory Effects	8
Resistance Development.....	9
Future of Antimicrobials.....	9
References	10

ROLE OF ADDITIVES IN FOOD

Food additives may be classified by one of six primary functions they serve: preservation, improvement in nutritional value, addition or replacement of color, addition or replacement of flavor, improvement in texture, or processing aids (Branen and Haggerty, 2002). The focus of this book is food additives that contribute to preservation, although some food antimicrobials also contribute to color stability (sulfites, nitrites) or flavor (nitrites and certain organic acids). Readers are referred to *Food Additives* for a more complete treatise of all additives (Branen et al., 2002).

Despite the recognized requirement for food additives, their toxicologic safety continues to be evaluated and is often questioned. Few new additives have been approved by regulatory agencies in recent years, and it is doubtful that many additional ones will be approved in the future. Although there is no question that the risk from an additive must be minimal, it is apparent that such risk must be balanced against the benefits of the use of such additives. For example, reduction in the number of food poisoning cases or reduced food loss resulting from spoilage are benefits provided by antimicrobial additives. Balancing the risks versus the benefits is not easy and requires extensive research about the usefulness and toxicologic safety of the additives in question. In very few cases are additives totally devoid of risk; thus, an assessment of the degree of acceptable risk is often required. Responsibility for determining if risks outweigh benefits for any particular additive is

with scientists, legislators, regulatory personnel, food processors, and consumers. It is essential that all involved in the decision process be acutely aware of the risks and benefits of all additives.

DEFINITION AND FUNCTION OF CHEMICAL FOOD PRESERVATIVES

Since prehistoric times, chemicals have been added to preserve freshly harvested foods for later use. Drying, cooling, fermenting, and heating have always been the primary methods used to prolong the shelf life of food products. Whereas some chemical food preservatives, such as salt, nitrites, and sulfites, have been in use for many years, most others have seen extensive use only recently. One of the reasons for increased use of chemical preservatives has been the change in the ways foods are produced and marketed. Today, consumers expect foods to be readily available year-round, to be “free” of foodborne pathogens, and to have a reasonably long shelf life. Although some improvements have been made using packaging and processing systems to preserve foods without chemicals, antimicrobial preservatives play a significant role in protecting the food supply (Davidson et al., 2002). In addition, because of changes in the marketing for foods to a more global system, products seldom are grown and sold locally as in the past. Today, foods produced in one area are often shipped to another area for processing and to several other areas for distribution. Several months or years may elapse from the time food is produced until it is consumed. To accomplish the long-term shelf life necessary for such a marketing system, multiple effective means of preservation are often required. It is important to note that, with rare exceptions, food antimicrobials are not able to conceal spoilage of a food product (i.e., the food remains wholesome during its extended shelf life). In addition, because food antimicrobials are generally bacteriostatic or fungistatic, they will not preserve a food indefinitely.

Food antimicrobials are classified as “preservatives.” Chemical preservatives are defined by the U.S. Food and Drug Administration (FDA; 21CFR 101.22(a)(5)) as “any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties.” Therefore, preservatives are used to prevent or retard both chemical and biological deterioration of foods. Those preservatives used to prevent chemical deterioration include **antioxidants**, to prevent autoxidation of pigments, flavors, lipids, and vitamins; **antibrowning compounds**, to prevent enzymatic and nonenzymatic browning; and **antistaling compounds**, to prevent texture changes. Those additives used to prevent biological deterioration are termed “antimicrobials.”

The FDA defines antimicrobial agents (21CFR 170.3(o)(2)) as “substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors.” The traditional function of food antimicrobials is to prolong shelf life and preserve quality through inhibition of spoilage microorganisms. However, antimicrobials have been used increasingly as a primary intervention for inhibition or inactivation of pathogenic microorganisms in foods (Davidson and Zivanovic, 2003). Surprisingly, few food antimicrobials have been used exclusively to control the growth of specific foodborne pathogens. Examples include nitrite inhibition of *Clostridium botulinum* in cured meats and, more recently, selected organic acids as spray sanitizers against pathogens on beef carcasses, nisin and lysozyme against *Clostridium botulinum* in pasteurized processed cheese, and lactate and diacetate to inactivate *Listeria monocytogenes* in processed meats (65FR17128, March 31, 2000).

Antimicrobials may be classified as traditional or naturally occurring (Davidson, 2001). The former are approved for use in foods by many international regulatory agencies (Tables 1.1 and 1.2). Naturally occurring antimicrobials include compounds from microbial, plant, and animal sources. They are, for the most part, only proposed for use in foods. A few, such as nisin, natamycin, lactoferrin, and lysozyme, are approved by regulatory agencies in some countries for application

TABLE 1.1
Traditional or Regulatory-Approved (U.S. Food and Drug Administration) Food Antimicrobials^a (Title 21 of the Code of Federal Regulations)

Compound(s)	Microbial Target	Primary Food Applications	CFR Designation
Acetic acid, acetates, diacetates, dehydroacetic acid	Yeasts, bacteria	Baked goods, condiments, confections, dairy products, fats/oils, meats, sauces	184.1005, 182.6197, 184.1754, 184.1185, 184.1721, 172.130
Benzoic acid, benzoates	Yeasts, molds	Beverages, fruit products, margarine	184.1021, 184.1733
Dimethyl dicarbonate	Yeasts	Beverages	172.133
Lactic acid, lactates	Bacteria	Meats, fermented foods	184.1061, 184.1207, 184.1639, 184.1768
Lactoferrin	Bacteria	Meats	GRAS Notice No. GRN 000067
Lysozyme	<i>Clostridium botulinum</i> , other bacteria	Cheese, casings for frankfurters, cooked meat, and poultry products	184.1550; GRAS Notice No. GRN 000064
Natamycin	Molds	Cheese	172.155
Nisin	<i>Clostridium botulinum</i> , other bacteria	Cheese, casings for frankfurters, cooked meat, and poultry products	184.1538; GRAS Notice No. GRN 000065
Nitrite, nitrate	<i>Clostridium botulinum</i>	Cured meats	172.160, 172.170, 172.175, 172.177
Parabens (alkyl esters (propyl, methyl, heptyl) of <i>p</i> -hydroxybenzoic acid)	Yeasts, molds, bacteria (Gram positive)	Beverages, baked goods, syrups, dry sausage	184.1490, 184.1670, 172.145
Propionic acid, propionates	Molds	Bakery products, dairy products	184.1081, 184.1221, 184.1784
Sorbic acid, sorbates	Yeasts, molds, bacteria	Most foods, beverages, wines	182.3089, 182.3225, 182.3640, 182.3795
Sulfites	Yeasts, molds	Fruits, fruit products, potato products, wines	Various

^a For meat products, food antimicrobials permitted by USDA Food Safety and Inspection Service are listed in the Code of Federal Regulations, Title 9, Section 424.21 and 424.22.

Source: Davidson and Harrison, 2002

to foods (Tables 1.1 and 1.2). Extensive reviews on natural antimicrobials may be found in Dillon and Board (1994), Sofos et al. (1998), Naidu (2000), and Davidson and Zivanovic (2003).

SELECTION OF ANTIMICROBIALS

It is not an easy process to select the appropriate preservation system for a particular food product. The target pathogen or spoilage microorganisms must be identified first, and then the possible preservation systems must be evaluated via model studies and studies in the food product in question. Generally, a combination of chemical preservatives and other preservation methods is needed (Leistner, 2000).

TABLE 1.2
Regulatory-Approved Food Antimicrobial Designations in the European Union (E Numbers) and in *Codex Alimentarius* (INS Numbers)

Compound	E or INS Number	Compound	E or INS Number
Sorbic acid	200	Natamycin	235
K sorbate	202	DMDC ^a	242
Ca sorbate	203	K nitrite	249
Benzoic acid	210	Na nitrite	250
Na benzoate	211	Na nitrate	251
K benzoate	212	K nitrate	252
Ca benzoate	213	Acetic acid	260
Ethyl paraben	214	K acetate	261
Na ethyl paraben	215	Na acetate	262
Propyl paraben	216	Na diacetate	262
Na propyl paraben	217	Ca acetate	263
Methyl paraben	218	Lactic acid	270
Na methyl paraben	219	Propionic acid	280
Sulfur dioxide	220	Na propionate	281
Na sulfite	221	Ca propionate	282
Na hydrogen sulfite	222	K propionate	283
Na metabisulfite	223	Boric acid	284
K metabisulfite	224	Na tetraborate	285
Ca sulfite	226	Na lactate	325
Ca hydrogen sulfite	227	K lactate	326
K hydrogen sulfite	228	Ca lactate	327
Nisin	234		

^a Dimethyl dicarbonate.

Source: Verbruggen, 2002.

Selection of the proper antimicrobial depends on several primary factors, including the spectrum of antimicrobial activity, the chemical properties of the antimicrobial, the physicochemical properties and composition of the food product in question, and the type of preservation or processing and storage systems used. Each of these factors is discussed in detail.

ANTIMICROBIAL SPECTRUM

The initial selection of the antimicrobial is normally based on an assessment of the overall microbial spectrum of the chemical in question. The antimicrobial spectrum should involve an evaluation of the compound against various types of microorganisms (e.g., bacteria, yeasts, molds) and forms of those microorganisms (vegetative cells vs. spores). Even species, strain, and Gram reaction (positive vs. negative) can have dramatic influences on apparent activity. For example, the activity of very hydrophobic antimicrobials may be limited against Gram-negative bacteria, which have the ability to screen the antimicrobials because of the outer membrane lipopolysaccharide layer. Quite often, a broad spectrum of activity, although desired, is not easy to achieve. Few chemicals have the ability to inhibit several different types, species, or strains of microorganism.

The antimicrobial spectrum of a compound is generally determined by following the growth of organisms in the presence of various concentrations of the antimicrobial. Appropriate methods for *in vitro* evaluation of the activity of food antimicrobials are described in Chapter 21, "Methods for Activity of Assay and Evaluation of Results." Seldom, however, does growth in a synthetic

microbiological medium parallel that in a food product. Thus, one must be wary of an antimicrobial spectrum determined only in a synthetic medium. The final confirmatory test for the antimicrobial spectrum and activity must be carried out in a food product because food components and properties can dramatically alter the overall spectrum and activity of the antimicrobial. As a rule, it is important to know the conditions under which any antimicrobial spectrum was determined before projections are made regarding the usefulness of an antimicrobial.

Probably the best method for determining what type of food antimicrobial to use would be based on its mechanism of action and/or target in the cell. However, the exact mechanisms through which antimicrobials affect microbial growth are complex and difficult to determine. Mechanisms of action of food antimicrobials generally are classified as reaction with the cell membrane, causing permeability changes or interference with uptake and transport, inactivation of essential enzymes, interference with genetic mechanisms, or inhibition of protein synthesis. Unfortunately, few targets, even for the regulatory-approved food antimicrobials such as organic acids, have actually been fully elucidated. If the mechanism of the compound is known, combinations of antimicrobials with different mechanisms could be used against the microorganisms in the food product (Davidson et al., 2002).

PHYSICOCHEMICAL PROPERTIES OF THE ANTIMICROBIAL

The overall microbial spectrum, the mode of action, and the efficacy of compounds are largely dependent on the chemical and physical properties of the antimicrobial. The polarity of a compound is probably the most important physical property. Water solubility or hydrophilic properties appear to be necessary to assure that the antimicrobial is soluble in the water phase, where microbial growth occurs (Robach, 1980). At the same time, however, antimicrobials acting on the hydrophobic cell membrane appear to require some lipophilic properties (Brannen et al., 1980). Thus, like emulsifiers, antimicrobials appear to require a specific hydrophile–lipophile balance for optimal activity. Hydrophilic properties appear necessary to allow water solubility where microbial growth occurs; lipophilic characteristics appear to be required to allow the antimicrobial to react with the membrane of the microorganisms. The balance needed in a synthetic medium, however, may differ significantly from that needed for a food product, owing to polarity of the food components.

The boiling point of a compound can also directly influence the activity of an antimicrobial, especially its carry-through properties. If a food is heated during processing, a highly volatile compound can be lost. Certain phenolic compounds, for example, are vaporized, and significant losses occur during a cooking process. High volatility can also result in a noticeable odor, which contributes to an off-flavor in a food product.

FOOD-RELATED FACTORS

The chemical reactivity of the antimicrobial with other food components can significantly affect activity. Reaction with lipids, proteins, carbohydrates, and other food additives can result in an overall decrease in the activity of the antimicrobial compound. Chemical reactions, in addition to decreasing antimicrobial activity, can also result in the formation of off-flavors, -odors, and -colors. Sorbic acid, for example, can be degraded by certain *Penicillium* species isolated from cheese to produce 1,3 pentadiene, which has a kerosene off-odor. A sensory evaluation is often needed to assure that antimicrobials do not directly or indirectly through chemical reaction alter the color, flavor, or texture of a food product.

Other factors leading to reduced effectiveness among food antimicrobials are food component interactions. Most food antimicrobials are amphiphilic. As such, they can solubilize in or be bound by lipids or hydrophobic proteins in foods, making them less available to inhibit microorganisms in the food product. Interaction with lipids probably results in the greatest interference with antimicrobial activity. Highly active antimicrobial compounds that are hydrophobic tend to partition

TABLE 1.3
pK_a of Regulatory-Approved Organic Acids

Compound or Group of Compounds	pK _a
Acetic acid	4.75
Benzoic acid	4.19
Lactic acid	3.79
Propionic acid	4.87
Sorbic acid	4.75

into the lipid areas of the food and away from the water phase, where microbial growth occurs (Branen et al., 1980; Rico-Munoz Davidson, 1983).

pH of the food can result in ionization of an antimicrobial and a change in activity. For example, organic acids function at low concentrations only in high-acid foods (generally less than pH 4.5 to 4.6). This is because the most effective antimicrobial form is the undissociated acid, which exists in the majority only at a pH below the pK_a of the compound. All regulatory-approved organic acids used as antimicrobials have pK_a values less than 5.0 (Table 1.3), which means their maximum activity will be in high-acid foods. For food products with a pH of 5.5 or greater, there are very few compounds that are effective at low concentrations. Although the undissociated form of a weak acid has most of the antimicrobial activity, Eklund (1983) demonstrated that the anion does contribute slightly to antimicrobial activity.

In the undissociated form, organic acids can penetrate the cell membrane lipid bilayer more easily. Once inside the cell, the acid dissociates because the cell interior has a higher pH than the exterior. Bacteria maintain internal pH near neutrality to prevent conformational changes to the cell structural proteins, enzymes, nucleic acids, and phospholipids. Protons generated from intracellular dissociation of the organic acid acidify the cytoplasm and must be extruded to the exterior. Because protons generated by the organic acid inside the cell must be extruded using energy in the form of adenosine triphosphate (ATP), the constant influx of these protons will eventually deplete cellular energy.

Chelating compounds such as ethylenediamine tetraacetic acid (EDTA) or its salts have a potentiating effect on some antimicrobials. They expand the activity of certain antimicrobials (e.g., nisin, lysozyme) to include Gram-negative bacteria, which are not normally inhibited by the compounds alone (Cutter and Siragusa, 1995; Branen and Davidson, 2004). In addition, some Gram-positive bacteria are more susceptible to certain antimicrobials in the presence of chelators including EDTA. It is theorized that chelators may destabilize the LPS layer of the outer cell membrane of the Gram-negative bacteria, allowing the antimicrobials access to the inner cell membrane.

PROCESS FACTORS

The type of preservation process used in conjunction with antimicrobials has a significant influence on the type and level of antimicrobial needed. Certain preservation processes may result in the need to control sporeformers that have the ability to survive the heating process. A lowering of the water activity can select for those organisms that have the ability to survive and/or grow at lower water activity. Generally, molds survive and yeasts can grow at a lower water activity than bacteria, thus indicating the need for a different antimicrobial. Refrigeration generally selects for psychrotrophic Gram-negative microorganisms, thus requiring an antimicrobial capable of limiting the growth and activity of these organisms.

Packaging can directly alter the environment of the food and thus influence the overall growth pattern and type of organisms in the food. Vacuum or modified atmosphere packaging results in

low oxygen tension, which inhibits growth of molds and several bacteria but allows certain facultative anaerobic microorganisms such as lactic acid bacteria to grow. A significant amount of research has been done on the addition or incorporation of food antimicrobials to packaging materials. This would allow inhibition of spoilage or pathogenic microorganisms at the surface of a packaged product.

The ability of certain antimicrobials to inhibit microorganisms can be overcome on extended storage. Depending on the time and temperature of storage, these antimicrobials can be volatilized or directly react with other food components. In some cases, the microbial flora may have the ability to metabolize the antimicrobial and thus, after an extended time, render it ineffective.

Generally, food antimicrobial compounds are primary contributors to a combination of inhibitors and inhibitory conditions (e.g., low pH, low temperature). This is sometimes termed “hurdle technology” (Leistner and Gorris, 1995; Leistner, 2000).

CONSIDERATIONS IN THE USE OF FOOD ANTIMICROBIALS

SANITATION

An antimicrobial is never a substitute for good sanitation in a food processing plant, and low microbial loads must always be sought. Few, if any, regulatory-approved antimicrobials are able to preserve a product that is grossly contaminated. In addition, although food antimicrobials will extend the lag phase or inactivate low numbers of microorganisms, their effects can be overcome. If the number of microorganisms contaminating a food product is high, significantly higher quantities of an antimicrobial may be needed.

TOXICOLOGIC SAFETY

Perhaps the most important aspect of any compound proposed for use as a food additive would be its toxicologic characteristics. It is obviously essential that an additive for use as a food antimicrobial be safe for human consumption. The safety of some food antimicrobials has been questioned over the years, which has led to limitations on their use. Although attempts have been made to use other preservation systems or to produce additive-free foods, it is unlikely that the current marketing system could exist without the use of antimicrobials. Requirements for toxicologic safety will limit the ability of the industry to develop new antimicrobials. The stringent requirements for toxicologic safety testing can result in costs in the millions of dollars before approval for a new additive can be obtained. Ultimately, of course, an antimicrobial must be nontoxic to test animals and humans, based on several studies. It is also important that the antimicrobial be metabolized and excreted by the body. The compound or its breakdown products should also not result in buildup of residues in body tissues. Valid assay methods for the antimicrobial are also a necessity so that levels can be easily followed. The majority of the antimicrobials now being used in food products have been extensively tested for toxicologic safety, and although questions will continue to be asked, an evaluation of the risks versus the benefits indicates that these antimicrobials are acceptable. The food industry will always be reluctant to expand the use of food preservatives, however, because of the unknown problems that may result from an increased consumption of such compounds in combination with other additives and food components. Certainly, however, the ability of an antimicrobial to contribute to the prevention of foodborne illness must be taken into account when assessing the overall safety of the individual antimicrobial.

A possible total or partial shift to naturally occurring antimicrobials is being investigated by many researchers. Because they occur in nature, it is often thought that naturally occurring antimicrobials are less toxic than synthetic compounds. This is not always true. A naturally occurring antimicrobial must be shown to be nontoxic either by animal testing or by its continuous consumption by consumers as a food over a long period. The latter may be problematic even for some

common potential natural antimicrobials such as spice extracts. This is because, although spices have been consumed for centuries, they are not normally consumed in the concentrations necessary to achieve antimicrobial activity. In addition to lack of toxicity, naturally occurring compounds must be able to be metabolized and excreted so as to not lead to residue buildup. Finally, they should be nonallergenic or bind or destroy important nutrients in a food product (Harlander, 1993).

LABELING

One of the alleged attractions of naturally occurring antimicrobials is their reduced negative impact on the labeling of foods. Consumers are reportedly concerned about the presence of synthetic chemicals in their foods and would prefer natural compounds. A potential problem with natural antimicrobials is that if they are highly purified, they may need to be approved as food additives. This would involve very expensive and time-consuming toxicologic testing. In addition, the compound would probably have to be listed using a chemical name on a food label. This, of course, would defeat the purpose of using a natural compound. For that reason, less purification may be better. If a product is simply an “extract of” a commonly consumed plant or animal food product, it is much less likely to require complex regulatory approval for use (Davidson and Zivanovic, 2003). This is only possible if the product from which the extract is taken is known to be nontoxic.

ECONOMICS OF USE

A food antimicrobial will not be useful to the food industry unless it is inexpensive enough and has the ability to pay for itself based on reducing spoilage and minimizing foodborne illness. Extensive studies at a pilot-plant level are necessary on any food additive to prove its overall usefulness. In many cases, an additional 2 or 3 days of shelf life can significantly help to offset the cost of using an antimicrobial. The efficiency of these compounds can be extremely important in determining the overall economics of their use.

ACTIVITY VALIDATION METHODS

Currently, there are few standardized methods for validation of the activity of regulatory-approved food antimicrobials. In the United States, there are methods for the determination of the activity of lysozyme (U.S. *Code of Federal Regulations*, 21CFR 184.1550) and nisin (U.S. *Code of Federal Regulations*, 21CFR 184.1538); there are no other activity assays specified or required for food antimicrobials. If antimicrobials are to be used exclusively as inhibitors of pathogens in food products, assays need to be developed that evaluate the activity of these compounds against the pathogen they are designed to kill. The reason for these assays is that various conditions of process or storage could reduce the effectiveness of the compound. For example, it is known that peptides, such as nisin, are susceptible to inactivation by enzymes in foods. Therefore, just as thermal processes need validation, so should there be validation for the activity of food antimicrobials.

SENSORY EFFECTS

Another major factor that needs to be addressed when applying antimicrobials is their potential impact on the sensory characteristics of a food. Many antimicrobials must be used at high concentrations to achieve activity against target microorganisms. Obviously, compounds that negatively affect flavor and odor or contribute inappropriate flavors and odors would be unacceptable. In addition to adverse effects on flavor, odor, or texture, it would be unacceptable for a food antimicrobial to mask spoilage because spoilage may protect consumers from ingesting foodborne pathogens.

RESISTANCE DEVELOPMENT

Because the activity spectra are often different for each antimicrobial, the microflora contaminating a food product significantly influences the choice of the antimicrobial needed. One should be cautious, however, not to select an antimicrobial solely according to its ability to control the predominant microorganism present. Because of their specificity, selecting antimicrobials that control some genera but not others may result in selecting for and creating favorable conditions for growth of other organisms. For example, phenolic compounds may inhibit certain Gram-positive food-poisoning bacteria, but because of the reduced activity against Gram-negative bacteria, favorable conditions can be created for growth and spoilage by these latter organisms.

Potential food antimicrobials should not contribute to the development of resistant strains nor alter the environment of the food in such a way that growth of another pathogen is selected. There has been much interest in the effect of environmental stress factors (e.g., heat, cold, starvation, low pH/organic acids) on developed resistance of microorganisms to subsequent stressors. Microorganisms exposed to a stress may become more resistant and have enhanced survival to subsequent stresses (Foster, 1995; Buchanan and Edelson, 1999). For example, it has been demonstrated that some bacterial pathogens may develop a tolerance or adaptation to organic acids following prior exposure to low pH. Although this increased resistance may be a problem in application of organic acids for controlling pathogens, it has not been shown to occur in an actual food processing system (Davidson and Harrison, 2002).

FUTURE OF ANTIMICROBIALS

Antimicrobials will undoubtedly continue to be needed to provide the food supply that will be demanded in the future. The global economy in which we live results in foods being transported throughout the world. If foods are to arrive in the condition expected, preservatives will be needed. This will also require the development of uniform worldwide regulations regarding the use of these chemicals in food products. It is hoped through the information presented in the following chapters that food scientists, regulators, and consumers will be better able to determine whether the risks outweigh the benefits for selected additives now available and for some compounds of potential future value. The information should also serve as a basis for selection of any new antimicrobial developed in the future.

The future of research in the area of food antimicrobials will likely be on two fronts. First is the expansion of information on the antimicrobial spectrum of natural antimicrobials. This research will be more focused on the appropriate use of natural antimicrobials or utilization of compounds in situations in which they are compatible. For example, certain compounds, such as thymol, carvacrol, and allyl isothiocyanate (AIT), are not compatible with certain foods. Appropriate or compatible use would involve using these compounds in foods in which they add to the positive sensory characteristics of the product in addition to improving food safety or increasing shelf life. A second major area of research involves use of antimicrobials in combinations with each other and with traditional or novel processing methods. To more effectively apply antimicrobials so that synergistic activity is possible will require knowledge of the mechanisms of action of the compounds. Attaining synergistic activity with antimicrobial combinations requires that the components have different mechanisms. In addition, natural antimicrobials will be increasingly looked on as adjuncts in hurdle technology and used with milder nonsterilizing, nonthermal processing methods such as high hydrostatic pressure or pulsed electric fields (Smid and Gorris, 1999).

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2 Sodium Benzoate and Benzoic Acid

John R. Chipley

CONTENTS

Introduction and Historical Background	11
Physical and Chemical Properties and Natural Occurrences.....	12
Mechanism of Action.....	12
Antimicrobial Activity	17
Spectrum of Action.....	17
Influence of Other Chemicals and Physical Environment	20
Acquired Resistance to Benzoic Acid.....	22
Microbial Metabolism of Benzoic Acid.....	26
Reaction with Food Constituents.....	28
Regulatory Status	29
Applications	29
Use in Various Food Systems.....	29
Use as a Postharvest Fungicide	32
Other Applications	33
Storage and Handling	34
Toxicology.....	34
Assay	37
Acknowledgments.....	38
References	38

INTRODUCTION AND HISTORICAL BACKGROUND

Benzoic acid is one of the oldest chemical preservatives used in the cosmetic, drug, and food industries. Sodium benzoate was the first chemical preservative approved for use in foods by the U.S. Food and Drug Administration (FDA) (Jay, 2000). Its preservative action appears to have been first described in 1875, when a relationship was established between the action of benzoic acid and that of phenol (Lueck, 1980). Because benzoic acid could not initially be produced synthetically in large quantities, it was not introduced for food preservation until around 1900 (Lueck, 1980). The advantages of its low cost, ease of incorporation into products, lack of color, and relatively low toxicity subsequently caused benzoic acid to become one of the most widely used preservatives in the world (Davidson, 2001).

During the last 10 years, several articles concerning various aspects of food additives and preservatives (e.g., benzoic acid) have been published. General evaluations of the use of these compounds in foods (Vogel, 1992), in prevention of microbial spoilage (Giese, 1994), in meat products (Gerhardt, 1995), in beverage manufacture (Giese, 1995), and in consumer attitudes toward the use of preservatives (Jager, 1994) serve as examples.

PHYSICAL AND CHEMICAL PROPERTIES AND NATURAL OCCURRENCES

Benzoic acid (C_6H_5COOH) and sodium benzoate (C_6H_5COONa) have the structural formulas shown in Figure 2.1. Benzoic acid (molecular weight 122.1), also called phenylformic acid or benzenecarboxylic acid, occurs in pure form as colorless or white needles or leaflets. It is soluble to a limited extent in water (0.18, 0.27, and 2.2 g dissolves in 100 ml water at 4°C, 18°C, and 75°C, respectively).

Sodium benzoate (molecular weight 144.1) is a white granular or crystalline powder. It is much more soluble in water than benzoic acid (62.8, 66.0, and 74.2 g dissolves in 100 ml water at 0°C, 20°C, and 100°C, respectively). For this reason, it is preferred for use in many cases. Potassium benzoate and calcium benzoate have also been approved for use, although their solubility in water is less than that of the sodium salt.

Benzoic acid occurs naturally in several foods and commodities (Table 2.1). It accounted for approximately 16% of the growth inhibition of *Saccharomyces bayanus* and *Pseudomonas fluorescens* from ethanolic extracts of cranberries (Marwan and Nagel, 1986a). Benzoic acid has also been identified as a major constituent in extracts of blackberries (Humpf and Schreier, 1991); of mushrooms, depending on the variety (Abdullah et al., 1994); and of fresh tomatoes (Marlatt et al., 1992).

Yogurts have been found to contain natural levels of benzoic acid (Stijve and Hischenhuber, 1984; Teuber, 1995). In an extensive review, Sieber et al. (1995) surveyed and analyzed many types of cultured dairy products and cheeses for the natural occurrence of benzoic acid (Table 2.2). Its presence appears to occur as a by-product of the microbial degradation of either hippuric acid or phenylalanine in these products (Figure 2.2). A third pathway, oxidation of benzaldehyde, may also contribute to benzoic acid generation. This may be of significance in countries like Switzerland, where benzoic acid has not been approved as a food additive (Sieber et al., 1989, 1990).

Benzoic acid has also been identified as a natural by-product in culture filtrates of *Lactobacillus plantarum* (Niku-Paavola et al., 1999). Several new types of low-molecular-mass compounds were also identified, and a mixture of these, along with benzoic acid, was found to have antimicrobial properties.

Naturally occurring nonflavonoid phenolic compounds, including derivatives of benzoic acid, were used to characterize commercial fruit juices (Fernandez de Simon et al., 1992). Orange, apple, pineapple, peach, apricot, pear, and grape juices were analyzed to establish phenolic profiles unique to each type of juice. In four of the seven juices, p-hydroxybenzoic acid was detected at 0.2 to 2.6 mg/l.

MECHANISM OF ACTION

It is the undissociated molecule of benzoic acid that is responsible for antimicrobial activity (Table 2.3). Gabel (1921) was one of the first to demonstrate that benzoic acid was effective against bacteria in acid media at a level of 0.1% and in neutral media at 0.2% but inactive in alkaline media. Similar results were reported for fungi and yeasts (Crues and Richert, 1929).

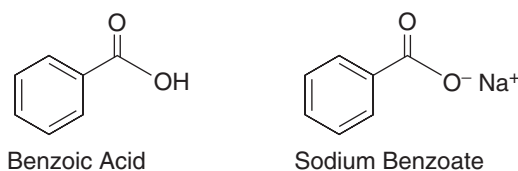


FIGURE 2.1 Structures of benzoic acid and sodium benzoate.

TABLE 2.1
Natural Occurrence of Benzoic Acid

Category	Product
Fruits and berries	Apples
	Apricots
	Berries (i.e., blackberries)
	Blueberries
	Cherries
	Cranberries
	Grapes
	Plums, greengage
	Prunes
	Strawberries
	Tomatoes
Fermented products	Beers
	Dairy, cultured
	Teas, black
	Wines
Spices and flavors	Cinnamon
	Cloves, ripe
	Licorice
Others	Coffee beans
	Honey
	Mushrooms
	Teas, green
	Tobacco

Note: Levels of benzoic acid vary (10 to 1000 mg/kg) depending on the food.

Source: Chipley (1993); Fenaroli (1996).

TABLE 2.2
Benzoic Acid Content of Cultured Dairy Products

Product	Range in Concentration (mg/kg)
Yogurts	9–56
Yogurt, fruit	5–39
Sour cream	10–18
Buttermilk	10–19
Cottage cheese	2–18
Cheese	0–200
Cheese, non-smear-ripened	0–41
Cheese, smear-ripened	0–622

Source: Adapted from Sieber et al. (1995).

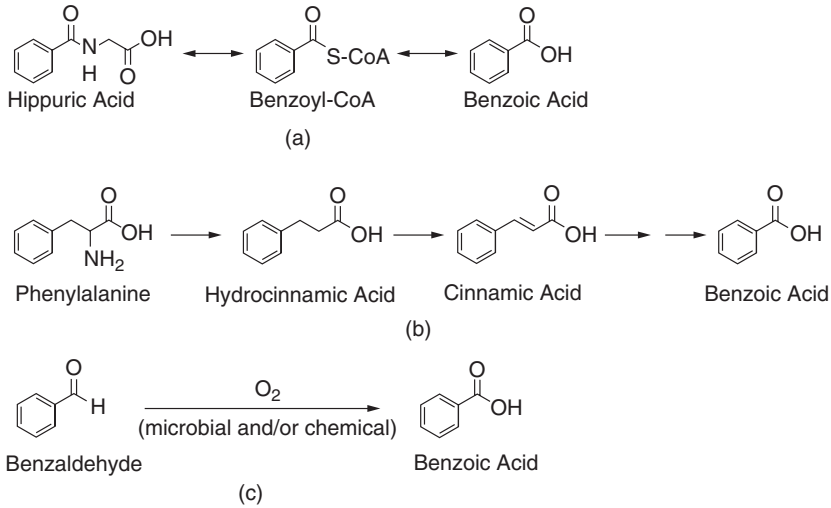


FIGURE 2.2 Biosynthesis of benzoic acid by lactic acid bacteria in fermented dairy products. Source: Adapted from Sieber et al. (1995).

TABLE 2.3
Effect of pH on the Dissociation
of Benzoic Acid

pH	Undissociated Acid (%)
3	93.5
4	59.3
5	12.8
6	1.44
7	0.144
pK	4.19

Source: From Baird-Parker (1980).

Rahn and Conn (1944) reported that the antimicrobial effect of benzoic acid was nearly 100 times as efficient in strongly acidic solutions as in neutral solutions, that only the undissociated acid was antimicrobial, and that the toxicity of sodium benzoate in solution was a result of the undissociated benzoic acid molecule.

Macris (1975) observed a rapid uptake of benzoic acid in *Saccharomyces cerevisiae*. Saturation was reached in about 2 minutes and remained constant thereafter. The strong dependence of uptake on pH was because of the relative distribution of undissociated and dissociated forms in solution, not to the pH itself. The undissociated form was the only one taken up by cells. The effect of temperature on the uptake was similar to that observed in enzymatic reactions. Proteinaceous material was apparently involved in the uptake of this preservative.

The effects of pH, NaCl, sucrose, and sorbic and benzoic acids on the growth of 30 strains of food spoilage yeasts have been reported (Praphailong and Fleet, 1997). *Zygosaccharomyces bailii* and *Yarrowia lipolytica* were the most resistant to the two preservatives at pH 5.0. Sorbate was more inhibitory than benzoate on the strains that were tested. The combined effects of pH and benzoate on the growth of these strains are presented in Table 2.4. As expected, increasing the pH of the culture medium decreased the effectiveness of this preservative.

TABLE 2.4
Effect of Benzoic Acid on the Growth of Some Important Food Spoilage Yeasts at Different pH Values

Yeast	No. of Strains Tested	Maximum Concentration (mg/L) Allowing Growth			
		pH 2	pH 3	pH 5	pH 7
<i>Debaromyces hansenii</i>	3	NG	—	500	1200
	1	—	—	250	1200
<i>Yarrowia lipolytica</i>	5	—	250	1200	1200
<i>Pichia anomala</i>	1	—	—	500	1200
<i>P. membranaefaciens</i>	2	—	—	750	1200
<i>Saccharomyces cerevisiae</i>	2	NG	—	750	1200
<i>Kluyveromyces marxianus</i>	4	NG	—	500	1200
<i>Kloeckera apiculata</i>	4	—	—	750	1200
<i>Zygosaccharomyces bailii</i>	6	NG	250	1200	NG
<i>Z. rouxii</i>	2	NG	—	750	1200

Note: NG, no growth in the absence of benzoic acid; — no growth in the presence of 250 mg of benzoic acid/L.

Source: Adapted from Praphailong and Fleet (1997).

The effects of benzoic acid were studied in the preservative-resistant yeast *Z. bailii* (Warth, 1991a). At concentrations up to 4 mM, fermentation was stimulated and low levels of benzoate were accumulated. Near the minimum inhibitory concentration (MIC) (10 mM), fermentation was inhibited, adenosine triphosphate (ATP) levels declined, and benzoate was accumulated to higher levels. The author concluded that the primary action of benzoic acid in *Z. bailii* was to cause a general energy loss (ATP depletion). Fermentation was inhibited as a result of high ATP usage rather than of lowered intracellular pH (Warth, 1991b). Inhibition of several glycolytic enzymes was not observed.

Burlini et al. (1993) preincubated cells of *S. cerevisiae* with benzoate or sorbate at an extracellular pH of 6.8, then added glucose. The resulting metabolic effects included reduced glucose consumption and suppression or reduction of glucose-triggered reactions within cells. The authors stated that, in general, the glucose-induced switch from gluconeogenesis to glycolysis was prevented. Growth rates were substantially reduced by both preservatives even at an extracellular pH of 6.8. The authors concluded that the metabolic effects of benzoate or sorbate were only marginally pH dependent.

Sorbic and benzoic acids both affected the heat shock response and thermotolerance of *S. cerevisiae* (Cheng and Piper, 1994). These effects were dependent on the pH of the culture medium. The authors reported that these preservatives were among the first compounds shown to act as selective inhibitors of heat-induced protein expression in yeasts.

Two modeling studies to predict the inhibitory effects of organic acid preservatives have been reported. In yeast (*S. cerevisiae*), Lambert and Stratford (1999) demonstrated the following:

1. Inhibition depends more on the preservative concentration within cells rather than on undissociated acid concentration per se.
2. Microbial cells can pump protons out during extended lag phase and raise internal pH despite further influx of preservative.

3. Duration of the lag phase can be predicted from the model.
4. Theoretical ATP consumption for proton pumping can be directly correlated with reduction in cell yield.

Hsiao and Siebert (1999) constructed a model based on physical and chemical properties of organic acids and principal components analysis. Acid-susceptible bacteria (*Bacillus* and *Alicyclobacillus*) and acid-resistant bacteria (*Lactobacillus* and *Escherichia coli*) were grown in media containing organic acids. MICs were determined for each acid and used with principal components to produce models with good correlations.

Only those organic acids that are lipophilic, such as benzoic acid, show antimicrobial activity. One hypothesis is that they inhibit or kill microorganisms by interfering with the permeability of the microbial cell membrane, causing uncoupling of both substrate transport and oxidative phosphorylation from the electron transport system (Freese et al., 1973). In *Bacillus subtilis* (Freese, 1978), membrane transport of amino acids is inhibited, resulting in nutritional starvation of cells (Gould et al., 1983; Gould, 1989). Inhibition of transport in turn results from the destruction of the proton-motive force caused by the continuous shuttle of protons into cells by benzoic acid (Davidson, 1997). The inhibitory potency of this acid can be determined by its lipid/water partition coefficient, its pK value (Table 2.3), and the ability of benzoic acid molecules to delocalize the negative charge of ions within the interior of cells and thus increase their membrane mobility.

More than 99% of the viable cells of *Listeria monocytogenes* were injured after exposure to a solution of 8.5% sodium benzoate (pH 7.0) for 1 hour (Buazzi and Marth, 1992). Metabolic injury was evident by the inability of cells to tolerate 6% NaCl in tryptose agar and the ability to grow on this medium with no added salt. Synthesis of messenger ribonucleic acid (mRNA) was critical for restoration of salt tolerance. Increased permeability of cell membranes during the course of benzoate injury was not observed.

The effects of organic acid preservatives on the growth and intracellular pH of *E. coli* have been reported (Salmond et al., 1984). The authors suggested that the potency of weak acids, such as benzoic, as food preservatives is related to their capacity specifically to reduce the intracellular pH. They also stated that although both the undissociated and dissociated forms of these acids cause the intracellular pH to fall, growth inhibition is the result predominantly of the undissociated acid. A pH-related increase in protein production was observed when cultures of *E. coli* were grown in liquid media with or without 20 mM benzoate (Lambert et al., 1997). At pH 6.5, benzoate addition resulted in an increased production of 33 proteins. Production of 12 of these was induced at pH 8.0 as well as pH 6.5.

Benzoate also inhibits amino acid uptake in *Penicillium chrysogenum* (Hunter and Segel, 1973), *B. subtilis*, *E. coli*, and *Pseudomonas aeruginosa* (Freese et al., 1973; Sheu et al., 1975; Eklund, 1980). In these studies it was suggested that the undissociated form of benzoic acid may diffuse freely through the cell membrane and then ionize in the cell, yielding protons that acidify the alkaline interior of the cell. Similar effects have been found using a variety of microorganisms, and the effectiveness of undissociated acids as preservatives has been noted for many years (Ingram et al., 1956; Corlett and Brown, 1980; Eklund, 1989; Davidson, 2001).

In addition to these mechanisms, benzoic acid or sodium benzoate can also inhibit specific enzyme systems within cells (Webb, 1966). For example, in many bacteria and yeasts, enzymes involved in acetic acid metabolism and oxidative phosphorylation can be inhibited (Bosund, 1960, 1962). In the citric acid cycle, α -ketoglutarate and succinate dehydrogenases appear to be quite sensitive (Bosund, 1962). The production of lipase by *P. fluorescens* was completely inhibited by sodium benzoate (Andersson et al., 1980). In resting cells of *E. coli*, 50% of the trimethylamine-N-oxide reductase activity was inhibited by 1.2 mM benzoic acid (Kruk and Lee, 1982). In yeast cells, phosphofructokinase activity was inhibited (Francois et al., 1986). Aflatoxin production by a toxigenic strain of *Aspergillus flavus* was greatly reduced by the presence of these compounds in synthetic media (Uraih and Chipley, 1976; Uraih et al., 1977; Chipley and Uraih, 1980). Reduction

was accompanied by the appearance of a yellow pigment that could be converted into aflatoxin B₁ by cell-free extracts. Conversion could be prevented by addition of benzoic acid or sodium benzoate. The results suggested that these preservatives blocked an enzymatic step late in the biosynthetic pathway of aflatoxin B₁.

With the previously mentioned results in mind, benzoic acid might also change the permeability of the microbial cell membranes by possibly causing a conformational change in the lip moieties of these structures. This might result in inhibition of membrane-bound enzymes as a secondary effect. Similar results were observed with 2, 4-dinitrophenol and cell envelope-associated enzymes of *E. coli* and *Salmonella* Enteritidis (Chiple, 1974).

Benzoate may also serve as a scavenger for free radicals (Harvath, 1979), as an inhibitor of D-amino acid oxidases (Quay and Massay, 1977; Yagi et al., 1980); as a weak inhibitor of poly(ADP ribose) polymerase (Oikawa et al., 1980), as an inhibitor of passive anion transport (Lucas-Heron and Fontenaille, 1979), and as an inhibitor of nitrosamine formation (Sung et al., 1988). It has been reported to have a flower-inducing effect in plants (Watanabe and Takimoto, 1979).

ANTIMICROBIAL ACTIVITY

SPECTRUM OF ACTION

Because the quantity of undissociated acid decreases with increasing pH (Table 2.3), the use of benzoic acid or sodium benzoate as a food preservative has been limited to those products that are acid in nature. Currently, these compounds are used primarily as antimycotic agents, and most yeasts and fungi are inhibited by 0.05% to 0.1% of the undissociated acid. Food-poisoning and spore-forming bacteria are generally inhibited by 0.01% to 0.02% undissociated acid, but many spoilage bacteria are much more resistant. Therefore, benzoic acid cannot be relied on to effectively preserve foods capable of supporting bacterial growth (Chichester and Tanner, 1972; Baird-Parker, 1980). MICs for some of the bacteria, yeasts, and fungi involved in food poisoning and food spoilage are given in Tables 2.5 and 2.6. Several factors interact to determine the MIC, including pH, temperature, genus and species of the microorganism in question, composition of the growth medium, prior exposure to the preservative, and environment from which the microorganism was originally isolated.

The sensitivity of 42 yeast cultures to sorbic and benzoic acids, potassium sorbate, and sodium benzoate was determined (Manganelli and Casolari, 1983). The scattering of MIC values was lower with potassium sorbate and benzoic acid and higher with sorbic acid and sodium benzoate. Addition of benzoate to chemostat cultures of *S. cerevisiae* decreased the biomass and increased the specific oxygen uptake rate of cells (Verduyn et al., 1992).

The effects of several preservatives and antimicrobial agents on aflatoxin B₁ production by *A. flavus* have been reported (Bauer et al., 1981). In liquid media, all treated cultures produced measurable levels of toxin 3 to 7 days later than controls. In benzoic acid-supplemented cultures, aflatoxin B₁ production was higher than in controls. The authors concluded that subinhibitory concentrations of these compounds may stimulate toxin production in some cases.

Sodium benzoate was found to control both growth and aflatoxin production by *Aspergillus parasiticus* in liquid media (El-Gazzar and Marth, 1987). Increasing the concentration of sodium benzoate increased the percentage of inhibition at the end of incubation (10 days). The average accumulation of mycelial dry weight was greater in the presence of benzoate than in its absence, however, with the greatest increase occurring when the medium contained 0.3% sodium benzoate.

Different concentrations of benzoic acid were tested to determine the effective levels capable of reducing the mycelial growth of six *Fusarium* and eight *Penicillium* species by 50% (Thompson, 1997). In general, *Fusarium* species were more sensitive to benzoic acid (210 to 420 µg/ml) than were *Penicillium* species (250 to 3000 µg/ml).

TABLE 2.5
Antimicrobial Spectrum of Benzoic Acid against Selected Bacteria,
Yeasts, and Fungi

Microorganisms	pH	MIC ^a
Bacteria		
<i>Bacillus cereus</i>	6.3	500
<i>Escherichia coli</i>	5.2–5.6	50–120
	6.0	100–200
<i>Klebsiella pneumoniae</i>	6.0	100–200
<i>Lactobacillus</i> sp.	4.3–6.0	300–1800
<i>Listeria monocytogenes</i>	5.6 (21°C)	3000
	5.6 (4°C)	2000
<i>Micrococcus</i> sp.	5.5–5.6	50–100
<i>Pseudomonas</i> sp.	6.0	200–480
<i>Pseudomonas aeruginosa</i>	6.0	200–500
<i>Staphylococcus aureus</i>	6.0	50–100
<i>Streptococcus</i> sp.	5.2–5.6	200–400
Yeasts		
<i>Sporogenic yeasts</i>	2.6–4.5	20–200
<i>Asporogenic yeasts</i>	4.0–5.0	70–150
<i>Candida krusei</i>		300–700
<i>Debaryomyces hansenii</i>	4.8	500
<i>Hansenula</i> sp.	4.0	180
<i>Hansenula subpelliculosa</i>		200–300
<i>Oospora lactis</i>		300
<i>Pichia membranefaciens</i>		700
<i>P. pastori</i>		300
<i>Rhodotorula</i> sp.		100–200
<i>Saccharomyces bayanus</i>	4.0	330
<i>S. cerevisiae</i>	4.0	600
<i>Torulopsis</i> sp.		200–500
<i>Zygosaccharomyces bailii</i>	4.8	4500
	4.0	1200
<i>Z. lentus</i>	4.0	500–1100
<i>Z. rouxii</i>	4.8	1000
Fungi		
<i>Alternaria solani</i>	5.0	1500
<i>Aspergillus</i> sp.	3.0–5.0	20–300
<i>Aspergillus parasiticus</i>	5.5	>4000
<i>Aspergillus niger</i>	5.0	2000
<i>Byssoschlamys nivea</i>	3.3	500
<i>Chaetomium globosum</i>	5.0	1000
<i>Cladosporium herbarum</i>	5.1	100
<i>Mucor racemosus</i>	5.0	30–120
<i>Penicillium</i> sp.	2.6–5.0	30–280
<i>Penicillium citrinum</i>	5.0	2000
<i>Penicillium glaucum</i>	5.0	400–500
<i>Rhizopus nigricans</i>	5.0	30–120

^a Minimum inhibitory concentration in µg/ml (ppm).

Source: Adapted from Chipley (1983, 1993); Davidson and Juneja (1990); Russell (1991); and Steels et al. (1999).

TABLE 2.6
Minimum Inhibitory Concentration of Benzoic Acid
for Food Spoilage Yeasts

Isolate ^a	MIC (ppm)
<i>Kluveromyces fragilis</i>	173
<i>Kloeckera apiculata</i>	188
<i>Pichia ohmeri</i>	200
<i>Hansenula anomala</i>	223
<i>Saccharomyces cerevisiae</i>	170–450
<i>Zygosaccharomyces rouxii</i>	242–330
<i>Zygosaccharomyces bisporus</i>	200–350
<i>Candida krusei</i>	440
<i>Saccharomycodes ludwigii</i>	500–600
<i>Schizosaccharomyces pombe</i>	500–567
<i>Zygosaccharomyces bailii</i>	600–1300

^a A total of 23 isolates were tested. Most were isolated from spoiled foods that had contained preservative. Isolates were grown at 25°C in yeast extract medium containing 5% glucose (pH 3.5) without addition of benzoic acid.

Source: Adapted from Warth (1989c).

Growth and aflatoxin production by toxigenic strains of *Aspergillus* were partially or completely inhibited by the undissociated form of six organic acid preservatives, including benzoic (Rusul and Marth, 1988). Salts, such as sodium and potassium chlorides and sodium nitrate, enhanced aflatoxin production when present at low levels but became inhibitory at higher levels.

Neosartorya fischeri is one of the most frequently isolated heat-resistant fungi causing spoilage of fruit juices and other heat-processed fruit-based products (Nielsen et al., 1989). Growth of this fungus was accompanied by production of fumitremorgin mycotoxins. Fungal growth was reduced by lowering the pH of laboratory media from 7.0 to 2.5; selected organic acids promoted growth and toxin production when added to the media. Small amounts (75 mg/L) of potassium sorbate or sodium benzoate completely inhibited germination of ascospores and subsequent outgrowth.

Both fungistatic and fungicidal properties have been attributed to benzoic acid, according to the results of a study involving several strains of *Trichophyton* and *Microsporium* (Pelayo, 1979). Sodium benzoate has been suggested as an inhibitor of cellulose-decomposing bacteria and fungi (Sauer, 1977).

Under appropriate conditions, bacteriostatic and bactericidal properties of benzoic acid can also be demonstrated. Beuchat (1980) reported that sodium benzoate (300 µg/ml) inhibited the growth of *Vibrio parahaemolyticus* in laboratory media and enhanced the rate of thermal inactivation of this organism at slightly higher concentrations. Ten generally regarded as safe (GRAS) substances, including benzoic acid, were tested against both the opaque and translucent morphotypes of *Vibrio vulnificus* (Sun and Oliver, 1995). Eight of these had a lethal effect on both morphotypes of this bacterium.

In a series of studies involving *L. monocytogenes* (El-Shenawy and Marth, 1988; Yousef et al., 1989), it was found that benzoic acid at concentrations of approximately 1000 to 3000 ppm had strong bacteriostatic, but relatively modest bactericidal, activities against cells in a liquid minimal medium. Incubation of cells in minimal media caused injury that depended on the temperature of incubation but not on the presence of benzoic acid. The authors questioned the suitability of benzoic acid alone to control this pathogen in foods. This organism was isolated from milk, and its survival

and growth were determined in media supplemented with organic acids and sodium benzoate (El-Shenawy and Marth, 1989). In general, inactivation or inhibition of growth occurred in inoculated media when a lower incubation temperature (13°C) and pH (5.0) were used and required lower levels of sodium benzoate. Combining glycerol monolaurate with benzoic acid gave greater inhibition of *L. monocytogenes* than when each preservative was tested alone (Oh and Marshall, 1994).

The effects of temperature, pH, sodium chloride, and three preservatives on the growth of three foodborne bacterial pathogens were studied using gradient gel plates (Thomas et al., 1993). Potassium sorbate was completely effective against Vero cytotoxigenic *E. coli* at all temperature/pH/NaCl combinations. It was also the most effective against *B. cereus*. At <25°C, sorbate was more effective than sodium benzoate against *S. aureus* when used with higher concentrations of NaCl. At 35°C, benzoate was the most effective preservative against *S. aureus*, especially when used at pH <6. Sodium nitrite was the least effective preservative tested. Increasing the acidity and/or NaCl generally improved the effect of all the preservatives.

Potassium sorbate and sodium benzoate did not have any significant effects on the heat resistance of four strains of *B. stearothermophilus* spores (Lopez et al., 1996). However, when these preservatives were added to recovery media, they were very effective inhibitory agents for heat-damaged spores (Lopez et al., 1998). Effectiveness increased as the pH of the recovery media decreased to 5.0.

An interesting method to determine concentrations of antimicrobial agents that provide infinite microbial stability has been developed (Marwan and Nagel, 1986b). This method was based on finding the relative effectiveness of an inhibitor, like benzoic acid, at various concentrations. The relative effectiveness values of benzoic acid were established for *S. bayanus* and *Hansenula* species. A plot of the inhibitor concentration versus the reciprocal of relative effectiveness was linear (Figure 2.3). The *x*-axis intercept was the concentration of the inhibitor that gave infinite microbial inhibition. The infinite inhibition concentrations for *S. bayanus* and *Hansenula* species were 330 and 180 ppm benzoic acid, respectively, when these organisms were grown in Trypticase® soy broth. Infinite inhibition concentrations could be affected by the growth medium. The growth patterns of *S. bayanus* in the presence of different concentrations of benzoic acid are shown in Figure 2.4. A typical dose-response effect may be observed.

INFLUENCE OF OTHER CHEMICALS AND PHYSICAL ENVIRONMENT

No antimicrobial is completely effective against all microorganisms present in a given foodstuff. In theory, one should be able to combine various antimicrobials having different modes of action to compensate for this deficiency. It should then be possible to achieve (1) a broader spectrum of action or (2) an increased antimicrobial action by using this combination (Lueck, 1980). For example, combinations of sorbic acid and benzoic acid inhibit several bacterial strains better than either antimicrobial alone (Rushing and Senn, 1963). Synergistic effects have also been reported using combinations of benzoate and sulfur dioxide, carbon dioxide, sodium chloride, boric acid, or sucrose (Rehm, 1960; Chichester and Tanner, 1972). Additive, synergistic, and antagonistic effects reported in several early studies have been extensively reviewed by Lueck (1980) for benzoic acid as well as for other preservatives. The influence of combinations of chemical antimicrobials on the growth of food-poisoning and spoilage microorganisms has been reported (Banks et al., 1988).

Individual and synergistic effects of pH, benzoic acid, and sorbic acid on the growth rate of the yeast *Z. bailii* were determined and expressed in polynomial equations (Cole and Keenan, 1986). Synergism between benzoic and sorbic acids was pH dependent. The anionic form of benzoic acid was shown to have a distinct effect on doubling time.

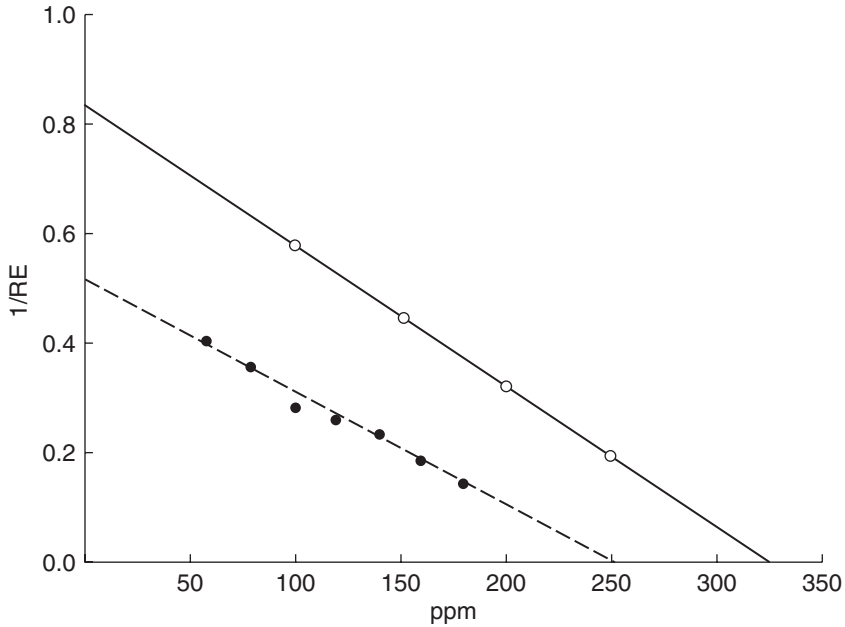


FIGURE 2.3 Effect of the medium on the inhibition of *Saccharomyces bayanus* by benzoic acid at 30°C and pH 4.0. **Open circles:** Trypticase® soy broth. **Closed circles:** yeast nitrogen base; RE, relative effectiveness (the time, in hours, it took the treated populations to reach a density of 70 Klett units divided by the time it took the control populations to reach the same value). Source: Marwan and Nagel (1986b).

Antagonistic as well as synergistic effects were observed when a wine yeast strain of *S. cerevisiae* was inoculated in a laboratory medium supplemented with several combinations of preservatives (Parish and Carroll, 1988). Sulfur dioxide was apparently antagonistic when used in combination with any of the other antimicrobial agents (including benzoate) tested in this study.

In addition to combining several preservatives together, it may also be advantageous to combine preservatives with physical methods of food preservation, such as heating, refrigeration, irradiation, or drying. Synergistic effects on yeasts and fungi using a combination of benzoic acid and heat have been reviewed by Lueck (1980) and have also been reported by Beuchat and Jones (1979) and Beuchat (1981a,b). The combined effects of preservatives and incubation temperature on biomass and patulin production by *Byssoschlamys nivea* in apple juice have been determined (Roland and Beuchat, 1984). Growth at 21°C, 30°C, and 37°C over a 25-day incubation period was significantly retarded by 75 ppm sulfur dioxide, 150 ppm potassium sorbate, and 500 ppm sodium benzoate. Sulfur dioxide had the most significant effect on the rate of biomass and patulin production by this organism, followed by potassium sorbate and sodium benzoate, respectively.

The effects of pH and benzoate on the growth of yeasts in an experimental grape drink containing 1.5 volumes of CO₂ have been reported (Kelly, 1975). Likewise, an experimental bottled beverage made from papaw fruit was reported to have a shelf life of 80 weeks at 10°C and 30°C when pasteurized and supplemented with sodium benzoate (1250 ppm and pH 3.9) (Okoli and Ezenweke, 1990). A combination of benzoic acid and low temperatures was used effectively to reduce the numbers of yeasts in grape juice (Pederson et al., 1961). Sodium benzoate prevented the growth of *Staphylococcus aureus* in an intermediate-moisture food (Boylan et al., 1976).

The presence of other chemicals may be beneficial or detrimental to the action of the preservative, depending on the chemical in question. For example, the antimicrobial activity of benzoic acid is reduced by nonionic surfactants (de Navarre and Bailey, 1956). However, benzoic acid can be solubilized with a surfactant to hold it in solution in its undissociated form (Lewis and Payne, 1958).

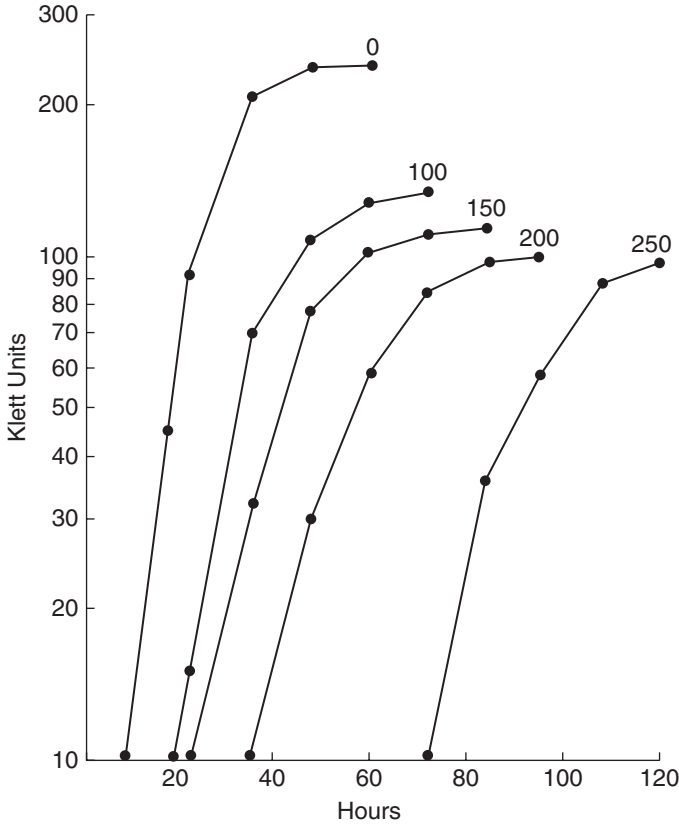


FIGURE 2.4 Inhibition of *Saccharomyces bayanus* by benzoic acid in Trypticase® soy broth at 30°C and pH 4.0. The concentrations of benzoic acid tested (in parts per million) are indicated. Source: Marwan and Nagel (1986b).

The effects of quinic acid on yeasts and molds were investigated (Kallio et al., 1985). Growth of yeasts was unaltered, but mold growth was accelerated in the presence of quinic acid. This acid was antagonistic to the antifungal effects of both potassium sorbate and sodium benzoate. Adding 1% quinic acid to media almost completely eliminated the inhibitory effect of 0.01% to 0.02% sodium benzoate. A combination of the polysaccharide chitosan (0.005%) and 0.025% sodium benzoate acted synergistically against three food spoilage yeasts suspended in saline solutions at pH 6.2 and 4.5 (Sagoo et al., 2002). At these pH values, the authors proposed that chitosan is highly polycationic and could react with the anionic components of the microbial surface; this might enhance the antimicrobial effects of benzoate.

ACQUIRED RESISTANCE TO BENZOIC ACID

There have been conflicting reports in the literature concerning the possibility of acquired resistance to benzoic acid by microorganisms. In a German study of 1959, cited by Lueck (1980), no resistance could be observed in cells of *E. coli* after 14 passages in media containing benzoic acid. However, recent studies imply that this may no longer be the case. The resistance of a wide variety of yeasts to benzoic acid has been reported in several studies. Carbonated and noncarbonated citrus drinks and fruit juices were involved. They had been supplemented with the maximum levels of benzoic acid permitted for use in these products.

An isolate of *Pichia membranaefaciens* from spoiled mango fruit was resistant to sodium benzoate (1500 and 3000 ppm) when grown in laboratory media at pH 4.0 and 4.5, respectively (Ethiraj and Suresh, 1988). However, this isolate was less resistant to potassium sorbate. The authors observed that preservative resistance may be strain specific. The heat-resistant yeast *Talaromyces flavus* was isolated from fruit juice concentrates involved in spoilage of packaged reconstituted fruit juice (King and Halbrook, 1987). Potassium sorbate and sodium benzoate prevented outgrowth, with the required concentrations lower when the pH of the growth media was reduced from 5.4 to 3.5.

Three strains of *T. flavus*, isolated from spoiled fruit products, were tested for tolerance to several organic acids during and after heating (Beuchat, 1988). Fumaric, sorbic, and benzoic acids were the most effective, and lethality was enhanced as the pH of the heating medium was reduced. The effects of sorbic and benzoic acids on the viability of ascospores varied depending on the strain and were influenced by other constituents in the heating medium. Heat resistance increased with age, and in some cases, heat tolerance appeared to be related to the morphology of the ascospores.

A strain of *S. cerevisiae* was found to have multiple resistances to both physical treatment and chemical preservatives (Guerzoni et al., 1990). In an inoculated peach-based system, the effectiveness of benzoic acid on this strain decreased as the water activity decreased and only a slight synergistic action with thermal treatments was observed.

Warth (1977) reported that cells of *Z. bailii* grew in the presence of high concentrations (600 mg/L) of benzoic acid at pH values below the pK of this acid (4.19). Resistance resulted primarily from an inducible energy-requiring system that transported benzoate from the cell. Because this system required energy, cells were resistant only when a sufficient energy source, such as glucose, was present. In a later study (Warth, 1988) it was determined that energy was required for the reduction in cytoplasmic benzoate concentration and for the maintenance of internal pH. Thus, the energy source was unavailable for growth, resulting in reduced growth yields and rates of cell production by yeasts. Several species of yeasts grown in the presence of benzoic acid tolerated 40% to 100% higher benzoic acid concentrations than yeasts grown in its absence (Table 2.7).

TABLE 2.7
Resistance of Yeast Species to Benzoic Acid

Isolate	MIC of Benzoic Acid (mg/L)	
	Cells Grown without Benzoic Acid	Cells Grown with Benzoic Acid ^a
<i>Saccharomyces cerevisiae</i>	100	170
<i>Kluveromyces fragilis</i>	125	173
<i>Kloeckera apiculata</i>	125	188
<i>Hansenula anomala</i>	140	223
<i>Saccharomyces cerevisiae</i>	175	285
<i>Candida krusei</i>	300	440
<i>Saccharomyces ludwigii</i>	300	650
<i>Schizosaccharomyces pombe</i>	325	567
<i>Zygosaccharomyces bailii</i>	600	1250

^a Adapted to benzoic acid by overnight growth in the presence of 0.25 mM benzoic acid (first five species) or 2 mM benzoic acid (last four species).

Source: Adapted from Warth (1988).

The genus *Zygosaccharomyces* is often regarded as being synonymous with food spoilage as a result of the remarkable resistance of its species to commonly used food preservatives such as sorbic, benzoic, and acetic acids and ethanol (Thomas and Davenport, 1985). *Z. bailii* can grow in concentrations of food preservatives in great excess of those normally, or legally, allowed in beverages and can adapt to concentrations even higher. The *Zygosaccharomyces* genus also contains some of the most osmotolerant species known, and most can grow at very low pH. Foods at particular risk are acidic and contain relatively high levels of sugar.

Uptake rates of benzoic and propanoic acids in cells of *Z. bailii* were proportional to the concentration of undissociated acid (Warth, 1989a). Benzoic acid was taken up 27 times faster by cells than propanoic acid. Glucose stimulated the rates of uptake and was also required for continuous elimination of benzoate from cells. The principal mechanism of uptake appeared to be diffusion of undissociated acid. Growth of yeasts in the presence of benzoic acid reduced their subsequent permeability to this acid. In another study (Warth, 1989b), growth of 22 isolates of 11 yeast species in the presence of benzoic acid enhanced their subsequent resistance to benzoic acid and other weak acid preservatives. The minimum pH for growth was not related to this acquired resistance. Growth in the presence of weak acid preservatives appeared to involve a common resistance mechanism.

Three selective media were compared for detecting and enumerating nine strains of *Z. bailii* grown in six commercial food products (Makdesi and Beuchat, 1996). These media were also evaluated for enumerating two of these strains grown in blueberry syrup with or without sodium benzoate (0, 300, and 600 µg/ml). The nonselective medium supported higher recovery of all nine strains from the six food products compared to the three selective media. Cells grown in syrup without sodium benzoate were more sensitive to selective media than were cells grown in syrup containing the preservative.

Mustard spoilage by *Z. bailii* has been described (Buchta et al., 1996). Eight tons of table mustard were exported to the United States. It was preserved using benzoic acid (1.5 g/kg) and packaged in glass jars. Spoilage resulted from a *Z. bailii* strain that was resistant to both benzoic and sorbic acids. A new osmophilic, preservative-resistant spoilage yeast, *Zygosaccharomyces lentus*, has been described (Steels et al., 1999). Five strains of this yeast grew over a wide range of temperature (4°C to 25°C) and pH (2.2 to 7.0). Growth at 4°C was significant, and all strains were osmotolerant. They were resistant to sorbic acid (≤ 400 mg/l) and benzoic acid (≤ 900 mg/l) at pH 4.0. Adaptation to higher preservative concentrations was demonstrated with benzoic acid. Resistance to dimethyldicarbonate was greater than that of *Z. bailii* and *S. cerevisiae*. The authors concluded that *Z. lentus* was likely to be more significant than *Z. bailii* in the spoilage of refrigerated foods.

Differences in resistance among yeast species could be the result of differences in the rate of penetration of the cell by benzoic acid, the capacity of the cell to remove it, or an intrinsic sensitivity to benzoic acid or its anion (Warth, 1989c). Resistant species may be less permeable than sensitive ones to undissociated benzoic acid (Warth, 1988). However, results also suggested that the resistance mechanism, in which benzoic acid is continuously removed from the cell, is a common and major determinant of the preservative tolerance of yeast species (Table 2.6) (Warth, 1989c).

Some yeasts are intrinsically resistant to relatively high concentrations of preservatives (Stratford and Lambert, 1999). *Zygosaccharomyces* and *Saccharomyces* are able to adapt to changing levels of preservatives such as benzoate. Adaptation can result in rapid growth in moderate levels or slow growth in previously inhibitory levels. The authors stated that microorganisms resist or adapt to weak acid preservatives (e.g., benzoate) by one of the following three methods:

1. Removal or metabolism. Uptake of weak acid preservatives by passive diffusion is quite rapid, requiring only 5 to 15 minutes to reach equilibrium. It is unlikely that removal of weak acids by metabolism could occur fast enough to alter the initial impact of these substances on spoilage yeasts.

2. Prevention of uptake. This appears unlikely because uptake occurs rapidly by passive diffusion. Whether efflux pumps are involved is open to debate.
3. Adaptation to or repair of damage. Several causes of damage by weak acid preservatives have been proposed, and adaptive mechanisms have been suggested. For example, if damage is caused by low intracellular pH, adaptation could involve enhanced removal of protons using H⁺ATPase. The authors proposed that some yeast adaptation to weak acid preservatives might simply be the result of accumulation of anions. However, earlier studies by Henriques et al. (1997) found that cells of *S. cerevisiae* were able to extrude benzoic acid by an energy-dependent mechanism. Piper et al. (1998) demonstrated the existence of a multidrug-resistance pump that actively extruded preservative anions from cells of this yeast.

In an extensive review, Brul and Coote (1999) described the modes of action of several types of preservative agents for foods and the microbial resistance mechanisms induced by these compounds. Weak organic acid preservatives have maximum inhibitory activity at low pH. This favors the uncharged, undissociated state of the molecule, which is freely permeable across the plasma membrane and is thus able to enter the cell. The inhibitory action is classically believed to occur when the molecule encounters the higher pH inside the cell and then dissociates, resulting in the release of charged anions and protons, which cannot cross the plasma membrane. The net result is that the preservative diffuses into the cell until equilibrium is reached in accordance with the pH gradient across the membrane. Anions and protons accumulate inside the cell. When this happens, inhibition of microbial cell growth occurs because various key metabolic reactions are inhibited, as cited previously (see “Mechanism of Action”). In yeasts, several workers have proposed that the actual inhibitory action of weak acid preservatives (benzoate and sorbate) could be the result of the induction of a stress response that attempts to restore homeostasis but results in the reduction of available energy pools needed for growth and other essential metabolic reactions (Warth 1991a, b; Holyoak et al., 1996; Henriques et al., 1997; Bracey et al., 1998).

Several microbial-resistance mechanisms induced by weak organic acid preservatives were also reviewed by Brul and Coote (1999). They included the acid tolerance responses of *E. coli* O157:H7 and *L. monocytogenes* and the role of membrane ATPase in the resistance of food spoilage yeasts. The authors proposed that in yeasts, simply pumping preservative anions out of cells could create a so-called “futile cycle” where the anions would reassociate at the lower external pH and reenter the cell. They assumed that any rate of diffusion across the plasma membrane remained unchanged and that cells would not alter their membrane composition or structure to reduce uptake of the toxic compound. However, they cited reports that adapted yeasts reduced the diffusion coefficient of preservatives across their plasma membrane so that transport of weak acids into cells was indeed reduced. Therefore, efflux of protons and anions by cells would not create a “futile cycle” if there was a concurrent reduction in the ability of weak acids to diffuse across the cell membrane and enter the cytosol.

Acquired resistance to benzoic acid has been reported for other microorganisms. Benzoic acid occurs naturally as a phytoalexin of immature apples (Seng et al., 1985). Mutants of the fungus *Nectria galligena*, which can infect apple tissues, were isolated and found to be resistant to benzoic acid. The pathogenicity of this fungus could be increased by growth on a medium supplemented with benzoic acid before inoculation into fruit.

MICs of sorbic and benzoic acids for *Gluconobacter oxydans* were 1000 and 900 mg/L, respectively, in media at pH 3.8. Reducing the pH of the media to 3.3 reduced the MIC of both antimicrobials by about 300 mg/L (Eyles and Warth, 1989). When *G. oxydans* was grown in the presence of sublethal concentrations of either compound before the MIC was determined, however, the MIC of both antimicrobials increased substantially within 1 hour.

Cells of *E. coli* initially grown in media at pH 5.0 survived exposures to inorganic acid or to acid pH plus organic acid that prevented subsequent growth of cells grown at pH 7.0 (Goodson

and Rowbury, 1989). Six organic acids, including benzoic, were evaluated; none of these acids inhibited the subsequent growth of acid-adapted cells. The authors proposed that acid-adapted organisms might survive in acid foods.

Eleven *E. coli* O157:H7 strains and four commensal strains of *E. coli* were tested for their ability to survive extreme acid exposures (Lin et al., 1996). Three previously reported acid-resistance systems were tested. They included an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system. When challenged at pH 2.0, the arginine-dependent system provided more protection to the O157:H7 strains than to commensal strains. However, the glutamate-dependent system provided better protection than the arginine system and appeared equally effective in all strains. Both of these systems were effective in protecting *E. coli* against the bactericidal effects of a variety of weak acids, including benzoic acid. The authors suggested that several acid-resistance systems might contribute to the survival of pathogenic *E. coli* in the gastrointestinal tract. They also found that once induced, acid-resistance systems remained active for prolonged periods of cold storage at 4°C.

Russell (1991) examined mechanisms whereby bacteria resist the effects of preservatives and nonantibiotic antibacterial agents. He stated that there were two types of bacterial resistance to preservatives. First, **intrinsic resistance** could result from a natural chromosomally controlled property of a bacterial cell. Examples of this type would include basic cell wall structure/organization and the possession of constitutive enzymes that enable bacteria to metabolize preservatives. Second, **acquired resistance** could result from genetic changes in a cell either by mutation or by acquisition of genetic material from another cell (usually by a plasmid). Resistance to preservatives caused by chromosomal gene mutation is an example. Others include the induction of acid resistance in *E. coli* O157:H7 (Lin et al., 1996) and protection of *L. monocytogenes* against lethal chemicals after adaptation to sublethal environmental stress (Lou and Yousef, 1997). Resistance to benzoic and sorbic acids is well documented in yeasts (Warth 1977, 1988). This is the result of an enhanced ability of adapted cells to eliminate these acids by energy-dependent extrusion. However, this mechanism has not yet been reported for bacteria. When considering ways of overcoming resistance or of preventing it, Russell (1991) noted that choosing a suitable preservative or a combination of preservatives might be the most effective.

MICROBIAL METABOLISM OF BENZOIC ACID

Johnson and Stanier (1971) stated that aerobic metabolism of benzoate by bacteria frequently proceeded through the β -ketoacid pathway (Figure 2.5). The enzymes of the pathway are inducible, and their synthesis is elicited by primary substrates or metabolic intermediates in the pathway.

In a taxonomic study, Moscoso-Vizcarra and Popoff (1977) found that Enterobacteriaceae could be divided into two groups with respect to benzoic acid metabolism. In the first group, benzoate could be metabolized through the β -ketoacid pathway; the second group consisted of those organisms that did not metabolize benzoate.

Thayer and Wheelis (1976) characterized an inducible benzoate permease system involved in the uptake of benzoate by cells of *Pseudomonas putida*. Metabolism of benzoate occurred by the

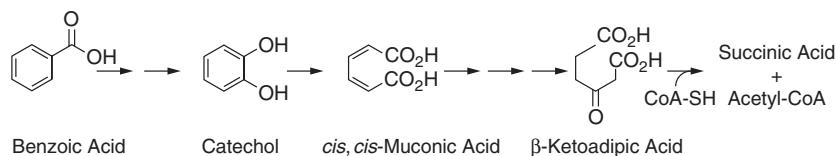


FIGURE 2.5 Proposed pathway for the aerobic microbial metabolism of benzoic acid. Source: Adapted from Zeyer and Kearney (1984).

β -keto adipate pathway. A mutant incapable of growth on benzoate was isolated and characterized. Active transport of benzoate may occur in this organism (Thayer and Wheelis, 1982).

Both chromosomal and plasmid-encoded pathways have been described for the catabolism of benzoate in *P. putida* (Jeffrey et al., 1992). In *Alcaligenes eutrophus*, a commonly occurring soil bacterium, the rate of benzoate metabolism may be regulated by the presence of succinate, end product of the β -keto adipate pathway (Figure 2.5) (Ampe et al., 1997). A permease-like protein involved in benzoate transport and degradation has now been isolated from cells of *Acinetobacter* (Collier et al., 1997).

Pseudomonas solanacearum, one of the causal agents of wilt in banana plants, was also capable of metabolizing benzoate (Kumari and Mahadevan, 1984). Catechol, *cis*, *cis*-muconate, and β -keto adipate were identified as intermediates of benzoate metabolism (Figure 2.5). These results were confirmed by assaying key enzymes from benzoate-grown cells. Kinetic models have been derived for the metabolism of [14 C]-benzoate by *Pseudomonas* species (Simkins and Alexander, 1984). Similar results have been reported with *Bacillus stearothermophilus* (Adams and Ribbons, 1988), with *Rhodococcus* (Janke et al., 1988), and with *Micrococcus* (Haribabu et al., 1984).

Hugo (1991) described the ability of nine species of *Pseudomonas* and *Vibrio* to degrade 18 aromatic compounds used as preservatives in medicines, foods, and cosmetics. He reported that benzoic acid was easily metabolized by all nine bacterial species tested. Seventeen strains of aerobic bacteria were tested for their ability to degrade 15 aromatic substrates (Lang, 1996). Benzoate was metabolized by 16 of these strains when added as the sole carbon source to liquid mineral media.

A variant of aerobic benzoate degradation has been found in a denitrifying bacterium (*Pseudomonas*) in which benzoyl-coenzyme A (CoA) is the first intermediate (Altenschmidt et al., 1993; Niemetz et al., 1995). This isolate was able to oxidize benzoate completely to carbon dioxide, both aerobically and anaerobically. Outlines of the new aerobic pathway were deduced. The proposed intermediates included benzoyl-CoA, 3-hydroxybenzoyl-CoA, gentisate, maleylpyruvate, fumarylpyruvate, and fumarate + pyruvate.

Harwood and Gibson (1986) stated that aerobic degradation of benzoate by various Gram-negative bacteria, including *Pseudomonas*, involves monooxygenase and dioxygenase enzymes that catalyze the incorporation of molecular oxygen into the aromatic nucleus to form dihydroxylated intermediates (see also Heider and Fuchs, 1997). Anaerobic metabolism can also occur but involves reductive saturation of the nucleus.

Compared to relatively well-characterized aerobic mechanisms for metabolism of benzoate and other aromatic compounds, anaerobic pathways have been far less studied and reported. One pathway proposed for the anaerobic microbial metabolism of benzoate is shown in Figure 2.6. Harwood and Gibson (1997) stated that this pathway could be divided into several phases: (1) formation of CoA thioesters, which are central intermediates in the anaerobic metabolism of many aromatic compounds (Elder and Kelly, 1994); (2) ring reduction; (3) introduction of a carbonyl group; (4) ring opening; and (5) a β -oxidation sequence. This is followed by conversion of the remainder of the molecule to acetyl-CoA. Nitrate, iron, and sulfate can be used as electron acceptors during metabolism (Colberg and Young, 1995).

Several actinomycetes were tested for the degradation of benzoate by growth in a liquid medium containing sodium benzoate (1 to 3 g/L) as the principal carbon source (Grund et al., 1990). Benzoate was converted to catechol by the strains able to grow on this substrate. Further metabolism proceeded via the catechol branch of the β -keto adipate pathway with the occurrence of high specific activities of catechol 1,2-dioxygenase.

In *Aspergillus niger*, benzoate and salicylate are metabolized via the protocatechuate and β -keto adipate pathways, respectively (Shailubhai et al., 1982). The enzymes of these pathways appeared to be under coordinate control and were repressed in the presence of a primary substrate, such as glucose. The enzymes responsible for ring cleavage were induced depending on the substrate used for growth. In the presence of benzoate, protocatechuate 3, 4-dioxygenase was induced, whereas salicylate induced catechol 1,2-dioxygenase for ring fission.

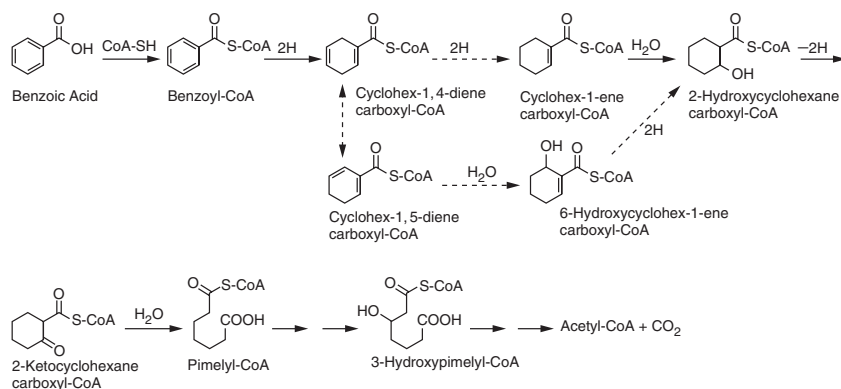


FIGURE 2.6 Proposed pathway for the anaerobic microbial metabolism of benzoic acid. Note: This sequence of reactions is compiled from several studies. Solid arrows indicate intermediates detected in benzoate-grown cultures. Dashed arrows indicate proposed intermediates. Source: Adapted from Koch et al. (1993); Harwood and Gibson (1997).

Microbial reduction of benzoate to benzyl alcohol under aerobic conditions has been reported (Kato et al., 1988). *Nocardia asteroides* and several fungi, including species of *Rhizopus* and *Mucor*, were found to have this capability. Although this appears to be a unique reaction occurring in microbial cells under aerobic conditions, the authors concluded that it may not be limited to a small number of microorganisms. However, Arfmann and Abraham (1993) found that benzoic acid as well as three hydroxyl derivatives could not be reduced to their respective alcohols when screened with a set of 20 microorganisms, most of which were fungi.

In the genus *Campylobacter*, one species, *C. jejuni*, has become recognized as a leading cause of human gastroenteritis (Hani and Chan, 1995). The N-benzoyl-glycine amidohydrolase (hippuricase) test is of paramount importance to differentiate *C. jejuni* (hippuricase positive) from other species. Currently, the hippuricase assay is the only reliable biochemical assay that can distinguish *C. jejuni* from *C. coli*. This enzyme cleaves hippuric acid into the constituent products glycine and benzoic acid. The authors isolated the gene responsible for the expression of hippuricase from 12 strains of *C. jejuni* and demonstrated that this gene was not present in 17 isolates of *C. coli*. They also showed that the gene was absent in other *Campylobacter* species. Based on these results, the authors proposed that a species-specific diagnostic DNA probe for human gastrointestinal infection caused by *C. jejuni* could be developed.

REACTION WITH FOOD CONSTITUENTS

The variability of the levels of benzoic acid in samples of commercial food products may indicate a preferential binding of this compound by certain food constituents. For example, Ganzfried and McFeeters (1976) developed a model system to evaluate binding of benzoic acid by lipids and proteins. Both bound benzoic acid to approximately the same extent. An equation was obtained to estimate the ratio of bound to unbound acid in food products based on their lipid and protein content. Heintze (1978) reported that benzoic acid accumulated in the lipid phase of herring salad, although stability was not adversely affected with time. Sodium benzoate has been shown to inhibit the growth of some of the microorganisms used for analyzing the vitamin content of foods (Voigt et al., 1979).

Models were tested in several food systems to determine the inhibition of 18 strains of *L. monocytogenes* by benzoic acid and other compounds (Ramos-Nino et al., 1996). In foods with high protein or lipid content, antilisterial activity was much lower than predicted, making the models unacceptable under these conditions.

In the early 1990s it was discovered that the combination of ascorbic acid and sodium or potassium benzoate in beverages could, under certain conditions, generate detectable levels of benzene. In one case (a fruit juice–mineral water combination), benzene levels higher than the permissible levels for mineral waters prompted a recall in one state. Subsequent studies indicated that soft drinks containing both ascorbic acid and sodium benzoate could produce very low levels of detectable benzene. Gardner and Lawrence (1993) demonstrated that low levels of detectable benzene could be produced in aqueous solutions of sodium benzoate and ascorbic acid in the presence of the transition metal catalysts iron (III) or copper (II). Although the detectable yield was extremely low (<1 ppb), the authors recommended that the combination of ascorbic acid and sodium benzoate in foods and beverages be evaluated more carefully. Similar results were reported by McNeal et al. (1993) when they analyzed several foods and fruit-flavored soft drinks for benzene.

REGULATORY STATUS

In the United States, benzoic acid and sodium benzoate are GRAS preservatives (Code of Federal Regulations, 1977/1988; Title 21, Secs. 184.1021 and 184.1733) up to a maximum permitted level of 0.1%. They may also be used in certain standardized foods. In most other countries, the maximum permissible quantities generally range between 0.15% and 0.25%. They may also include foods from which benzoate is excluded in the United States. An ADI (average daily intake) value of 0 to 5 mg/kg of body weight has been specified for benzoic acid (Pollard, 1990).

A *Food Technology* special report (1986) reviewed the characteristics, applications, and limitations of preservatives and antimicrobial agents, including benzoic acid. Potassium benzoate is now commercially available and apparently evolved in response to consumers' interest in reduced sodium intake. Regulatory agencies consider it to be GRAS to the same extent that sodium benzoate is GRAS as a preservative with the limitation of 0.1%. The potassium salt is less water soluble (42.4 g per 100 ml solution) than the sodium salt. Approximately 1.1 times more potassium salt than sodium salt is required to obtain the same level of benzoic acid in solution and the same level of antimicrobial effect. Product information for both salts is available (Miles Laboratories, Inc.). Calcium benzoate has also been approved for use, although its solubility in water is much less than the sodium salt (Pollard, 1990).

APPLICATIONS

Benzoic acid and sodium benzoate are most suitable for foods and beverages that naturally are in a pH range below 4.5 or that can be brought into this range by acidification. As food preservatives, their main advantages include low price, ease of incorporation into products, and lack of color. However, the narrow pH range in which they are effective, the off-flavor they may impart to the foods they are to preserve, and their toxicologic properties compared to some other preservatives have all contributed to recent efforts by food processors to replace benzoic acid and sodium benzoate with other preservatives with better characteristics. Benzoate does not control the growth of high levels of microorganisms and therefore cannot be used to compensate for poor ingredients or processing.

USE IN VARIOUS FOOD SYSTEMS

Benzoic acid and sodium benzoate have been widely used to preserve beverages, fruit products, bakery products, and other foods in several countries (Tables 2.8 and 2.9). Applications of these preservatives to foods have been reviewed by Chichester and Tanner (1972), Lueck (1980), Kimble (1977), and Chipley (1983, 1993). In addition, the use of benzoic acid and other chemicals in the processing and freezing of shrimp has been reviewed (Chandrasekaran, 1994).

TABLE 2.8
Maximum Permitted Levels (ppm) for Benzoic Acid and Its Salts in Foods
in Selected Countries

Food	Canada	Denmark	Norway	Sweden	U.K.	U.S. ^a
Fish products	1000					
Fish, semipreserved	1000	1000	5000	2000		1000
Fruit juice	1000	200	1500	1000	800	1000
Fruit pulp	1000		1500	1000	800	1000
Jam, jellies	1000	500	1500	1000		1000
Liquid egg white, yolk or whole		500	5000	10,000		
Margarine, salted	1000			2000		1000
Mayonnaise		1000	3000	2000		1000
Mustard	1000	1000	3000	2000		1000
Pickles, relishes	1000	1000	1500	2000	250	1000
Salads, salad dressings	1000	1000	1500	2000	250	1000
Sauces, ketchup	1000	1000	1500	2000	250	1000
Soft drinks containing fruit juice	1000	200	1500	1000	800 ^b	1000
Soft drinks, carbonated	1000	200	500	1000	160 ^c	GMP ^d

^a In the United States, a maximum level of 1000 ppm benzoic acid or its salts may be used for all products listed. A maximum level of 2000 ppm may be used in orange juice for manufacturing. Orange juice not for manufacturing may not contain a preservative (federal standards of identity).

^b Soft drinks for consumption after dilution.

^c Soft drinks for consumption without dilution.

^d Good manufacturing practice.

Source: Adapted from Chipley (1993).

Recent reports indicate that these preservatives continue to be of benefit in a variety of products. Fresh catfish were dipped in various concentrations of sodium benzoate or potassium sorbate and smoked, then evaluated during shelf life (Efiuvwevwere and Ajiboye, 1996). Sorbate treatment was more effective than benzoate and increased the shelf life by 8 days.

Halotolerant fungi were isolated from salted dried fish (Chakrabarti and Varma, 2000). *Aspergillus flavus*, *A. niger*, and *Penicillium* sp. were dominant. *Penicillium* sp. was most sensitive to propionic acid and sodium benzoate, but *A. flavus* was most sensitive to potassium sorbate. Propionic acid (0.06%) or potassium sorbate (0.02%) or sodium benzoate (0.04%) inhibited growth of all three fungi.

Several additives and preservatives were evaluated as inhibitors of melanosis (black spot) and microbial spoilage in prawns in chilled storage (Montero et al., 2001). A combination of sodium benzoate and kojic acid was effective in inhibiting melanosis. However, 4-hexylresorcinol was the most effective inhibitor of both melanosis and microbial spoilage in prawns. The shelf life of sardines stored in an acid brine containing 0.3% sodium benzoate was three times longer than that of the controls (Ponce de Leon et al., 1994).

The effectiveness of sodium benzoate and potassium sorbate, separately or combined, was evaluated against the growth of *Z. bailii* in an inoculated salsa mayonnaise stored at room temperature (Wind and Restaino, 1995). Potassium sorbate was more effective than sodium benzoate. However, neither preservative, separately or combined, prevented spoilage of the product by *Z. bailii*.

TABLE 2.9
Maximum Permitted Levels (ppm) for Benzoic Acid and Its Salts in Foods in the European Union^a

Commodity	Food	Level for Use
Beverages	Nonalcoholic flavored drinks (excluding dairy-based drinks)	150
	Spirits with <15% alcohol by volume	200
	Alcohol-free beer in keg	200
	Grape juice, unfermented	2000
	Liquid tea concentrates	600
Fish	Semi-preserved fish products (including fish roe)	2000
	Salted, dried fish	200
	Shrimp, cooked	2000
Fruits and vegetables	Low-sugar jams, jellies, marmalades, and other fruit-based spreads	500
	Candied fruits and vegetables	1000
	Vegetables in vinegar, brine, or oil	2000
	Prepared salads	1500
	Olives and olive-based preparations	500
	Aspic	500
Sauces	Emulsified sauces with fat content $\geq 60\%$	500
	Emulsified sauces with fat content $< 60\%$	1000
	Nonemulsified sauces	1000
Other	Nonheat-treated dairy-based desserts	300
	Confectionery (excluding chocolate)	1500
	Chewing gum	150
	Seasonings, condiments, mustard	1000
	Liquid egg white, yolk, or whole egg	5000
	Liquid dietary food supplements	2000
	Dietetic foods (excluding foods for infants and young children)	1500

^a The European Union (EU) is currently composed of 12 member nations. In the EU, benzoic acid and its sodium, potassium, and calcium salts are all permitted for use (E210–E 213) (Pollard, 1990).

Source: Adapted from Anon. (1995).

The microbial stability and quality of tomato juice and fermented cucumbers and carrots were improved by addition of a combination of sorbate and benzoate or benzoate alone (Bizri and Wahem, 1994; Fleming et al., 1996; Montano et al., 1997). The levels of some nutrients were reduced when tomato juice was treated with dimethyl dicarbonate. Tomato concentrate could be kept for up to 12 months at room temperature with the addition of salt, acetic acid, sodium benzoate, and potassium sorbate (Uboldi Eiroa et al., 1995). No viable microorganisms, including *Z. bailii*, were observed in any samples containing preservatives.

The effects of sodium benzoate and potassium sorbate on the thermal death rates of ascospores from the heat-resistant mold *N. fischeri* were evaluated in fruit juices (Rajashekhara et al., 1998). Comparable rates were noted when each preservative or the combination of both was used in mango juice heated to 85°C. In grape juice, potassium sorbate was more effective than sodium benzoate or their combination.

Growth and control of four *Salmonella* serotypes in a soft Hispanic type cheese were evaluated (Kasrazadeh and Genigeorgis, 1994). The minimum temperature that allowed growth was 8°C. Growth and control of two strains of *E. coli* O157:H7 were also evaluated in this cheese (Kasrazadeh and Genigeorgis, 1995). The minimum temperature that allowed growth of the *E. coli* strains was 10°C. In both studies, models were developed relating lag time and specific growth rate to

temperature. Growth was either prevented or delayed by adding sodium benzoate (0.3%) to cheese (pH 6.6) or adding potassium sorbate (0.3%) to cheese (pH 6.0) made from milk acidified to pH 5.9 with propionic acid.

Chicken skin was inoculated with *Salmonella* spp., *L. monocytogenes*, *C. jejuni*, or *S. aureus*, and wash solutions were evaluated for effectiveness in decontaminating the skin (Hwang and Beuchat, 1995). Washing the skin with solutions of either 0.3% or 0.5% lactic acid combined with 0.05% sodium benzoate reduced the numbers of these pathogens compared to washing with water. No viable cells from any of the four pathogens were detected on skin washed with lactic acid/benzoate solutions and stored for 8 days at 4°C. These solutions could be used for dips to sanitize chickens intended for frying before presentation to consumers (Hathcox et al., 1995).

Listeria monocytogenes is recognized as a foodborne pathogen of major concern to humans. Large outbreaks of infection have been linked to consumption of contaminated coleslaw, cheeses, milk, hot dogs, and luncheon meats. Contamination of many of these foods frequently occurs after processing. Application of antimicrobials as sprays or mists to meats following processing may be more effective than their addition in the meat formulation. The antimicrobial can be applied directly onto the product surface where cells of *L. monocytogenes* usually attach following cooking and during slicing and packaging (Farber and Peterkin, 1999; Tompkin et al., 1999). A study was conducted to evaluate aqueous dipping solutions of organic acids or salts to control *L. monocytogenes* on sliced, vacuum-packaged bologna stored at 4°C for up to 120 days. No significant increase in the numbers of this pathogen occurred on bologna slices treated with 2.5% or 5% acetic acid, 5% sodium diacetate, or 5% potassium benzoate for 120 days (Samelis et al., 2001). Sorbates and benzoates are currently approved in various countries for use as dipping solutions to prevent fungal growth in dry sausages (Sofos, 1989).

Combinations of organic acids, low pH, and ethanol were very effective bactericidal treatments for *L. monocytogenes* (Barker and Park, 2001). Benzoate was one of the most effective compounds tested in the presence or absence of ethanol. Ethanol-enhanced killing correlated with damage to the bacterial cytoplasmic membrane.

Unpasteurized apple cider has been implicated in several foodborne disease outbreaks caused by pathogenic microorganisms. Most research efforts have been directed toward *E. coli* O157:H7 in cider because of the severity of illness this pathogen causes, especially in younger children and the elderly. Survival of *E. coli* O157:H7 in apple juice and apple cider with or without potassium sorbate or sodium benzoate has been investigated. Both preservatives reduced the heat resistance of *E. coli* O157:H7, but benzoate was about two to eight times more effective than sorbate (Splittstoesser et al., 1996; Dock et al., 2000). At 8°C, the combination of sorbate and benzoate was more effective than either preservative alone (Zhao et al., 1993). Resistance to these preservatives was greater at 4°C than at 25°C (Fisher and Golden, 1998). These strains grew well in unpasteurized and pasteurized apple juice; growth was inhibited by benzoate or sorbate (Koodie and Dhople, 2001). Control strains of *E. coli* failed to grow in either type of apple juice. However, the growth of some O157:H7 strains in apple cider was not affected by the presence of either preservative (Miller and Kaspar, 1994).

A preservative treatment was developed that was capable of achieving the FDA mandate for a 5-log reduction of *E. coli* O157:H7 in apple cider (Comes and Beelman, 2002). The treatment that was successful included addition of 0.15% fumaric acid and 0.05% sodium benzoate followed by holding at 25°C for 6 hours and at 4°C for 24 hours. The same preservatives added to cider resulted in a greater than 5-log reduction in less than 5 and 2 hours when held at 25°C and 35°C, respectively.

USE AS A POSTHARVEST FUNGICIDE

Because of the long and successful use of benzoic acid in the processed food industry, this antimicrobial was evaluated for control of postharvest diseases of various fruits and vegetables. However, several factors may influence the effectiveness of benzoic acid in the treatment of fresh

fruits and vegetables, with perhaps the most pertinent consideration the pH of the superficial tissues (Eckert, 1967).

A benzoic acid-based polymer coating for apples has been developed (Ivanov et al., 1989). A mixture of fruit-coating polymers and potassium sorbate or sodium benzoate completely inhibited postharvest fungal growth on bananas (Al Zaemey et al., 1994). The shelf life of sliced apples and potatoes was extended by 1 week when they were dipped in a polysaccharide/protein edible coating containing sodium benzoate or potassium sorbate (Baldwin et al., 1996). Methylcellulose was mixed with chitosan and 4% sodium benzoate or potassium sorbate to produce a food-grade film (Chen et al., 1996). Significant antifungal properties were demonstrated against food spoilage fungi.

Benzoic acid and its derivatives have been proposed for use as fungicides, especially against toxigenic strains of *A. flavus*, in peanuts (Uraih and Offonry, 1981). It is currently used in animal feeds and in some tobacco products as a fungicide at levels of up to 0.1% and 0.025%, respectively.

OTHER APPLICATIONS

Benzoic acid is one of the oldest preservatives in the cosmetic and pharmaceutical industries. Generally, concentrations of 0.1% to 0.5%, incorporated as sodium benzoate, have been used to preserve cosmetic formulations. Currently, the cosmetic applications for benzoic acid have largely been taken over by more potent antimicrobial agents and compounds that are active over a wider pH range (Manowitz, 1968). A summary of the antimicrobial activities of 30 preservatives, including benzoic acid, used in cosmetics has been published (Bach et al., 1990). In the pharmaceutical industry, benzoic acid was used for oral preparations in concentrations of 0.05% to 0.1%. Although its use in pharmaceuticals has largely been displaced by other compounds (Grundy, 1968), possible areas for specialized applications still exist — for example, as a disinfectant for artificial kidneys (Kolmos, 1976), as a control for dentureborne *Candida albicans* (Lambert and Kolstad, 1986), and as a preservative in certain brands of cough syrup (Chen et al., 1988; Hewala, 1994).

The minimum inhibitory concentrations of sodium benzoate and dichlorobenzyl alcohol for 115 strains of dental plaque microorganisms were determined (Ostergaard, 1994). Sodium benzoate did not inhibit growth of any Gram-positive cocci. However, saliva samples from volunteers who used a dentifrice containing these antimicrobials indicated that for 5 to 10 minutes after toothbrushing, their concentrations were high enough to inhibit growth of periodontal pathogens. A single oral rinse with a mouth rinse containing these antimicrobials did not affect plaque removal (Danielsen et al., 1996).

A sodium benzoate–sorbic acid combination used in a pharmaceutical product for treating ulcers was effective against a wild strain of *Pseudomonas cepacia*, following official methods of analysis (Zani et al., 1997). However, this preservative system was ineffective against an adaptive-resistant strain. A p-hydroxybenzoate-based system was effective in protecting the product against a variety of strains of *P. cepacia* grown under different conditions.

Sodium benzoate has also been used to inhibit postharvest changes in fruits and vegetables. For example, Wang and Baker (1979) found that chilling injury to cucumber and sweet pepper fruits could be reduced by addition of 10 mM sodium benzoate as a 5-minute dip before chilling. The chilling-induced production of ethylene in cucumbers was inhibited by the addition of sodium benzoate (Wang and Adams, 1980).

Patents have also been issued for the use of benzoic acid or sodium benzoate as an industrial fungicide in several products. These include animal feedstuffs; varnishes; modified starch adhesives; adhesives for paper, textiles, and leather; lubricants; liquid coolants; rubber manufacturing; sealing gaskets for food containers; insoles for shoes; wood preservatives; and treatment for microbial infections in trees.

STORAGE AND HANDLING

Sodium benzoate should be stored in a cool, dry place. Containers should be kept closed as much as possible. This product is not corrosive. There is little danger of its being a toxicant or fire hazard under normal conditions of use. However, both benzoic acid and sodium benzoate are moderately toxic by ingestive, intramuscular, and intraperitoneal routes (Sax and Lewis, 1989).

TOXICOLOGY

Several toxicologic studies involving different animal species have been conducted using both benzoic acid and sodium benzoate. These have been summarized by exposure category in Table 2.10. In addition, Sax and Lewis (1989) reported data for several animal species based on the route of administration (Table 2.11). Extensive human feeding trials conducted early in the twentieth century led Chittenden et al. (1909) and Dakin (1909) to conclude that sodium benzoate was not deleterious to human health.

Benzoate does not appear to be accumulated in the body. The apparent reason for this involves a detoxifying mechanism whereby benzoate is absorbed from the intestine and “activated” by linkage with CoA to yield benzoyl coenzyme A. The overall reaction sequence proceeds as shown in Figure 2.7 (White et al., 1964).

The first reaction is catalyzed by a synthetase enzyme; the second is catalyzed by an acyltransferase enzyme. Hippuric acid, synthesized in the liver, is then excreted in the urine (White et al., 1964). Its formation can be increased greatly by administration of benzoic acid. Excretion of benzoic

TABLE 2.10
Toxicity of Benzoic Acid and Sodium Benzoate by Exposure Category^a

Category	Species Tested	Time Period	Level	Results
Acute	Rat		1.7–4.0 g/kg body weight	50% mortality
	Guinea pig, ^b rabbit, cat, dog		1.4–2.0 g/kg body weight	100% mortality
Subchronic	Mouse	3 months	80 mg/kg body weight	Mortality rate increase
	Mouse	5 days	3% of diet	50% mortality
	Mouse	3 months	4% of diet (as sodium benzoate)	No effect
	Human	3 months	1 g/day	No effect
	Human	14 days	12 g per 14 days	No effect
	Human	60–100 days	0.3–4.0 g per 60–100 days	No effect
	Human	Several days	5–10 g for several days (as sodium benzoate)	No effect
Chronic	Mouse	17 months	40 mg/kg body weight per day	Growth disturbance
	Rat	18 months	40 mg/kg body weight per day	Growth disturbance
	Rat	2 weeks	5% of diet (as sodium benzoate)	100% mortality
	Rat		1.5% of diet	Decreased growth rate
	Rat	2 weeks	1% of diet (as sodium benzoate)	No effect

^a Route of administration was peroral.

^b Route of administration was per os.

TABLE 2.11
Toxicity of Benzoic Acid and Sodium Benzoate by Route of Administration

Benzoic Acid				Sodium Benzoate			
Species Tested	Route of Administration ^a	Toxicity Results ^b	Level mg/kg	Species Tested	Route of Administration ^a	Toxicity Results ^b	Level mg/kg
Human	Skin	TDL ₀	6	Rabbit	Oral	LDL ₀	1994
Human	Oral	LDL ₀	500	Rabbit	Scu	LDL ₀	2000
Rabbit	Skin	MLD	500 mg ^c	Dog	Oral	LDL ₀	2018
Rabbit	Eye	SEV	100 mg ^c	Rat	Oral	TDL ₀	44000 ^d
Rabbit	Oral	LDL ₀	2000	Rat	Oral	LD50	4070
Rabbit	Scu	LDL ₀	2000	Rat	Ipr	TDL ₀	3000 ^e
Dog	Oral	LD50	2000	Mouse	Ims	LD50	2306
Cat	Oral	LD50	2000	Guinea pig	Ipr	LDL ₀	1400
Rat	Oral	LD50	2530				
Mouse	Oral	LD50	1940				
Mouse	Ipr	LD50	1460				
Guinea pig	Oral	LDL ₀	2000				
Guinea pig	Ipr	LDL ₀	1400				

^a Scu, subcutaneous; Ipr, intraperitoneal; Ims, intramuscular.

^b TDL₀, lowest published toxic dose; LDL₀, lowest published lethal dose; LD50, lethal dose 50 percent kill; MLD, mild irritation effects; SEV, severe irritation effects.

^c Total dosage.

^d Total dosage for 22 days to pregnant rats.

^e Total dosage for 3 days to pregnant rats.

Source: Adapted from Sax and Lewis (1989).

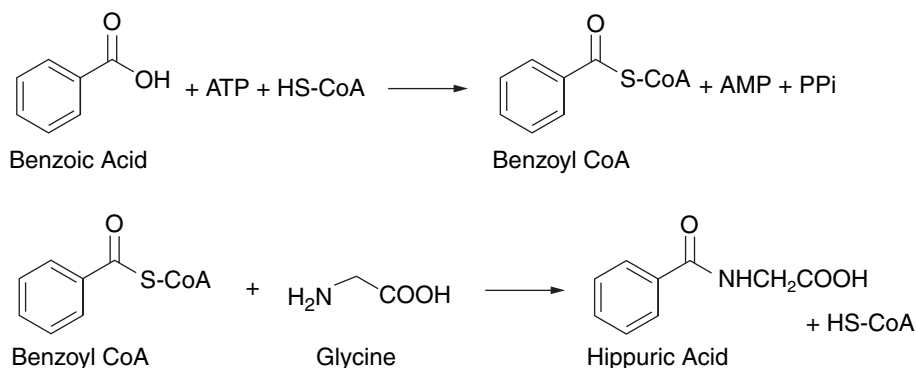


FIGURE 2.7 Metabolism of benzoic acid by mammals. Source: White et al. (1964).

acid by this particular pathway was first reported by Dakin (1909). Griffith (1929) found that this mechanism accounted for 66% to 95% of benzoic acid fed in studies in which the quantities were far in excess of those that might be ingested from foods preserved with permitted levels of benzoate. He also suggested that the remaining portion of the benzoate not excreted as hippuric acid may have been detoxified by conjugation with glucuronic acid and excreted in the urine by this route.

Akira et al. (1994) used deuterated benzoic acid and nuclear magnetic resonance spectroscopy to monitor metabolism in a healthy male volunteer. A single oral dose of 250 mg of benzoic acid was quantitatively metabolized to hippuric acid and excreted in the urine within 4 hours. The amount of sodium benzoate absorbed by human subjects may be estimated by determining the amount of hippuric acid excreted in the urine (Fujii et al., 1991).

Since the late 1970s, benzoic acid has been used in the treatment of patients with congenital errors of urea synthesis to develop an alternative pathway of nitrogen waste excretion (Kubota and Ishizaki, 1991). These authors analyzed plasma concentrations of benzoic and hippuric acids and urinary levels of hippuric acid after oral doses of sodium benzoate (40, 80, or 160 mg/kg) were given at least 1 week apart to 6 healthy male volunteers. The authors reported that both the biotransformation of benzoic acid to hippuric acid and the urinary excretion of hippuric acid followed first-order reaction rates.

These results have been confirmed in several animal studies. Different animal species differ in their ability to metabolize benzoic acid. This may be related to differences in the ability of liver and kidney cells to carry out glycine and glucuronic acid conjugation (for review, see Chipley, 1993).

Sodium benzoate inhibited the synthesis of glucose from lactate and generation of urea from ammonia when added to suspensions of rat hepatocytes (Cyr et al., 1991). Inhibition was caused by accumulation of benzoyl CoA with a resultant depletion of free CoA and acetyl CoA. Acceleration of the conversion of benzoyl CoA to hippurate by the addition of glycine restored the levels of free CoA and acetyl CoA and the rates of gluconeogenesis and ureagenesis.

In guinea pigs, the route of administration significantly affected the efficiency of benzoic acid metabolism (Nathan et al., 1990). When applied topically, only a small portion (6.9%) of the absorbed benzoic acid was conjugated with glycine to form hippuric acid. However, it was excreted almost completely as hippuric acid after systemic administration.

S. cerevisiae was used as a model system for testing antioxidant or prooxidant properties of benzoic acid and other weak organic acid food preservatives (Piper, 1999). Cell sensitivity to these acids was enhanced by aerobic rather than anaerobic growth conditions. The food preservatives were shown to have a strong prooxidant effect on aerobically grown yeast cells. They were also mutagenic to the yeast mitochondrial genome but only in the presence of oxygen. The author proposed that large-scale consumption of these preservatives in the human diet might generate oxidative stress within the epithelia of the gastrointestinal tract.

Some adverse reactions to benzoate ingestion have been reported (Schaubsluger et al., 1991). In an oral provocation test with 29 patients, the release of histamine and prostaglandin from mucosa was significantly increased by sodium benzoate exposure. In addition to occasional hypersensitivity reactions, severe anaphylaxis may occur (Michils et al., 1991). Sax and Lewis (1989) list benzoic acid as a human skin and eye irritant. They also cited data that indicated that sodium benzoate may be both an experimental teratogen and a mutagen.

The concentrations of several preservatives were determined and daily intakes were estimated in a survey of Japanese foods (Ishiwata et al., 1997). The estimated daily intake of benzoic acid was 11 mg/person based on the consumption of foods analyzed in this survey. Consumption of nonalcoholic beverages containing benzoic acid accounted for about 87% of the daily intake. Benzoic acid and its salts have been given a recommended average daily intake level of 0 to 5 mg/kg of body weight (Pollard, 1990).

A medical study was conducted to determine the effect of regular intake of cranberry juice, a natural source of benzoic acid (Table 2.1), on bacteriuria and pyuria in elderly women (Avorn et al., 1994). Subjects were randomly assigned to consume 300 ml/day of a standard cranberry beverage or a placebo drink for 6 months. At the end of the study, bacteriuria with pyuria was found in 28% of urine samples in the placebo group but in only 15% of the group randomized to the cranberry beverage. The findings suggested that use of a cranberry beverage reduced the frequency of bacteriuria with pyuria in older women. The authors stated that beliefs about the effects of cranberry juice on the urinary tract might have microbiological justification.

Several excellent reviews of the toxicologic and safety aspects of food additives used as antimicrobials or antioxidants have been written. For example, Parke and Lewis (1992) stated that the main toxic effects of benzoic and sorbic acids were various allergic responses in humans. In their opinion, steady increases in the incidences of bacterial food poisoning in Europe during the previous decade along with the emergence of "new" bacterial pathogens indicated that misplaced concern for potential toxicity of preservatives had resulted in increasing rather than decreasing health risks to consumers. These authors also described a procedure known as COMPACT (computer-optimized molecular parametric analysis of chemical toxicity). This procedure predicted if food additives were potential substrates of the cytochrome P450 family of enzymes and therefore susceptible to becoming potential carcinogens. Both benzoic and sorbic acids had COMPACT ratios that were moderate. For further information, the reader should also consult the proceedings of a World Health Organization conference (Anon., 1996) and two Spanish reviews of benzoic and sorbic acids (Frias et al., 1996).

ASSAY

Because benzoic acid is volatile in steam, it can be quantitatively isolated by acid steam distillation from foods. Occasionally, extraction with organic solvents may be advisable (Lueck, 1980; Nishiyama et al., 1995).

Two early collaborative studies should be noted. The first involved spectrophotometric determination of benzoate in ground beef (Maxstadt and Pollman, 1980). Reversed-phase, high-performance liquid chromatography was used in the second study to quantitate sodium benzoate in three types of soda beverages (Woodward et al., 1979). Both methods were adopted as official first action. Benzoic acid in human plasma and urine has been quantitated using a gas-liquid chromatographic technique (Sioufi and Pommier, 1980). Concentrations of 10 ng/ml could be detected in both substances. Microbiological assays for benzoic acid have also been reported. One procedure used two different genera of yeasts, and the minimum detectable concentration of benzoic acid was approximately 100 ppm (Ellerman, 1977).

A novel impedance-splitting method was evaluated for the microbial analysis of benzoic and sorbic acids in selected foods (Kroyer and Futschik, 1996). *E. coli* was used as the test organism, parameters were optimized, and quantitative analysis of these preservatives in various food extracts

TABLE 2.12
Recent Developments in Detection and Quantitation Methodologies for Benzoic Acid

Method ^a	Food	Reference
Various analytical methods	Fruits, fruit juices, citrus juices, orange juice (as adulterant)	Boland (1991, 1999)
HPLC	Yogurt	Serrano-Olea et al. (1991)
GC-MS	Various	Kakemoto (1992)
HPTLC	Beverages	Khan et al. (1994)
Reversed-phase HPLC	Various	Chen and Fu (1995)
SIM/GC-MS	Various beverages and foods	de Luca et al. (1995)
Liquid chromatography	Orange juice (as adulterant)	Lee (1995)
Reversed-phase HPLC	Packaged vegetables	Montano et al. (1995)
Capillary chromatography	Various beverages and foods	Pant and Trenerry (1995)
HPLC	Various	Mannino and Cosio (1996)
NMR spectroscopy	Orange juice (as adulterant)	Vogels et al. (1996)
Capillary electrophoresis	Various	Waldron and Li (1996)
Spectrophotometric/GLC/TLC	Various	Fazio (1999)
UV spectrophotometry	Topical ointments	Mahalanobis et al. (1990)

^a HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; HPTLC, high-performance thin-layer chromatography; SIM, selected ion monitoring; NMR, nuclear magnetic resonance; GLC/TLC, gas-liquid chromatography/thin-layer chromatography; UV, ultraviolet.

was performed. Advantages of the method were noted, and it was recommended for screening purposes.

The use of impedance microbiology to study effects of environmental factors on fungi has been reported by Nielsen (1991). Three isolates of the heat-resistant fungus *Neosartorya fischeri* were tested for their resistance to sodium benzoate. Inhibitory effects were strongly affected by pH, inoculum amount, and temperature. In addition, a biosensor prepared from tyrosinase-containing mushroom tissues has been developed and used to assay for the presence of benzoic acid (Wang et al., 1996).

Procedures for both qualitative and quantitative determination of benzoic acid and sodium benzoate can also be found in the Association of Official Analytical Chemists' *Official Methods of Analysis* (AOAC, 2002). Recent developments in detection and quantitation methodologies for several food products are outlined in Table 2.12.

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3 Sorbic Acid and Sorbates

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CONTENTS

Introduction	49
Chemistry	50
Antimicrobial Activity	54
Inhibition	54
Selective Action	55
Degradation	57
Interactions	57
Applications	61
Dairy Products	61
Vegetable Products	63
Fruit Products	64
Bakery Products	65
Meat Products	65
Miscellaneous Food Products	67
Other Applications	67
Mechanisms of Action	68
Toxicology and Safety	71
Detection and Analysis	73
Regulatory Status	74
References	75

INTRODUCTION

The antimicrobial and preservative properties of sorbic acid were first discovered, both in Germany and in the United States, by E. Miller and C. M. Gooding in the late 1930s and early 1940s, respectively (Lück, 1976, 1980; Sofos and Busta, 1981). The compound was first isolated as naturally occurring from the oil of unripened rowanberries (sorb apple or mountain ash tree) by the German chemist A. W. von Hoffmann in 1859 in London (Sofos, 1989). The first U.S. patent on sorbic acid was awarded in 1945 to C. M. Gooding and Best Foods, Inc. for their discovery that the compound was a good fungistatic agent for application to foods and food wrappers (Gooding, 1945).

Sorbic acid became available commercially during the late 1940s and early 1950s when testing as a preservative agent increased. Studies of that period also examined the biological properties and established guidelines for the safety of the compound. These developments resulted in the extensive use of sorbic acid and its salts as preservatives of foods and other materials throughout the world (Lück, 1976, 1980; Sofos et al., 1979a; Sofos and Busta, 1981; Sofos, 1989). Research efforts during the late 1950s and in the 1960s dealt mostly with the mechanism of sorbic acid

activity against microbial growth and the potential application of the compound in additional food products (Sofos and Busta, 1981).

During the 1970s extensive research was performed on the potential for using sorbic acid and its salts as antibotulinal agents in meat products, especially in combination with reduced nitrite levels for a reduction in nitrosamine formation (Sofos et al., 1979a; Sofos, 1981, Robach and Sofos, 1982), while the health effects of sorbate were reexamined (Sofos, 1981, 1989; Robach and Sofos, 1982).

Currently, sorbic acid and its more water-soluble salts, especially potassium sorbate, are collectively known as sorbates and are used widely throughout the world as preservatives for various foods, as animal feeds, as pharmaceuticals and cosmetics, and in other industrial applications (Sofos, 1989). The extensive use of sorbates as preservatives is based on their ability to inhibit or delay growth of numerous microorganisms, including yeasts, molds, and bacteria. There is variation, however, in inhibition of microorganisms by sorbates, depending on differences in microbial types, species and strains, substrate properties, and environmental factors. Under certain conditions, some microbial strains are resistant to inhibition by sorbate or even metabolize the compound. In general, however, sorbates are considered effective food preservatives when used under sanitary conditions and in products processed using good manufacturing practices.

The mechanisms of antimicrobial activity of sorbates are not fully defined (Sofos and Busta, 1981; Sofos et al., 1986; Sofos, 1989). Inhibition of bacterial spore germination (Sofos et al., 1979d) by sorbate is believed to occur at the connecting reactions of the germination process, probably through action on spore membranes or protease enzymes involved in germination (Blocher and Busta, 1985; Sofos et al., 1986). Inhibition of microbial metabolic function is probably the result of morphologic alterations in the structure of the cells, changes in the genetic material, alterations in cell membranes, and inhibition of enzymes or transport functions (Sofos, 1989).

CHEMISTRY

Sorbic acid is a straight-chain, trans-trans unsaturated fatty acid (2,4-hexadienoic acid; $\text{CH}_3\text{-CH=CH-CH=CH-COOH}$), with a molecular weight of 112.13 (Table 3.1). The carboxyl group of sorbic acid is highly reactive and results in formation of various salts and esters. The conjugated double bonds of sorbic acid are also reactive and can be influential in its antimicrobial activity as well as on the quality and safety of food products (Wedzicha and Brook, 1989; Sofos, 1989). The volatility of the compound in steam is useful in its isolation for quantitative detection in foods or other materials.

Sorbic acid forms colorless flakes or needles when crystallized, it is available as a white free-flowing powder or as granules, and it has a weak but characteristic acrid odor and acid taste. Commercially available salts include calcium, sodium, and potassium sorbates. Potassium sorbate may be manufactured as a powder or granules and, on an equivalent weight basis with the acid, has an antimicrobial potency of 74%. Sodium sorbate is a white fluffy powder and is commercially available as an aqueous solution that is sensitive to oxidation and stable for only a few weeks (Lück, 1980). The calcium salt is formed as a white odorless and tasteless powder.

The solubility of sorbic acid (Table 3.2) in water at room temperature is only 0.15 g per 100 ml, but it increases with temperature, the pH of the solution, or both. The solubility of sorbic acid is also higher in alcohols, especially ethanol, and in glacial acetic acid. The salts (e.g., potassium sorbate) of sorbic acid may find more frequent applications in foods because of their greater solubility in water. Calcium sorbate has a water solubility of 1.2% and is insoluble in fats, which makes it valuable as a delayed-release form of sorbic acid (Sofos, 1989, 1992). The sodium salt has a water solubility of 32% (wt/vol). The molecular weight of potassium sorbate is 150.22, and it constitutes the most soluble form of sorbate (Table 3.2). In food systems the solubility of the compound is estimated as higher than 50%. Good solubility (58.2% w/v at 20°C), stability, and

TABLE 3.1
Physical and Chemical Properties of Sorbic Acid and Potassium Sorbate

Property	Sorbic Acid	Potassium Sorbate
Chemical name	2,4-Hexadienoic acid	2,4-Hexadienoic acid potassium salt
Molecular formula	CH ₃ -CH=CH-CH=CH-COOH	CH ₃ -CH=CH-CH=CH-COOK
Molecular weight	112.13	150.22
Flash point, °C (COC ASTM D-92)	126	—
Ionization constant at 25°C	1.73 × 10 ⁻⁵	—
Density at 20°C g/ml	—	1.36
Melting range, °C	132–137	Decomposes higher than 270°C
Heat of combustion at 25°C, Btu/Lb	11,927	—
Alkalinity/acidity	—	ml 0.1 N NaOH to 0.8 ml 0.1 N HCL per 1.1 g
Vapor pressure, mm Hg, at		
20°C	<0.001	—
120°C	10	—
140°C	43	—
Boiling point, °C		
760 mm Hg	Decomposes	—
50 mm Hg	143	—
10 mm Hg	119	—
Purity, %	>98	>98
Water content, maximum%	0.5	1.0
Heavy metal content, maximum ppm	10	10
Arsenic content, maximum%	3	3
Ash, maximum%	0.2	—

Source: From Sofos (1989).

ease of manufacture make potassium sorbate the most widely used form in food systems (Sofos, 1989, 1992).

The amount of sorbic acid in the aqueous phase is reduced in food systems of higher lipid content because the solubility of sorbic acid in fat is approximately three times that in water (Gooding et al., 1995). The partition quotient is increased from 3.0 to 7.0 with increased levels (50%) of soluble food components, such as sugars and salts (Sofos and Busta, 1981). In general, the partitioning of sorbic acid between the water and fat phases of foods depends on the pH of the food, the type and amount of fat, and other ingredients present (Sofos, 1989). Aqueous solutions of sorbates are unstable and degrade through oxidation, whereas in the pure dry form they are stable. Oxidation of sorbic acid yields various carbonyl compounds, such as crotonaldehyde, malonaldehyde, acetaldehyde, acrolein, formic acid, malonic acid, and β-carboxylactolein (Arya and Thakur, 1988; Sofos, 1989). The rate of oxidation in aqueous solutions is increased at lower pH values by the presence of light and acids or increased temperatures. Oxidation and losses of sorbic acid are inhibited by antioxidants, appropriate packaging materials, and anaerobic conditions (Sofos, 1992). Ethylenediamine tetraacetic acid (EDTA) enhances sorbate degradation probably as a result of iron scavenging from the packaging material (glass or polypropylene) through EDTA complexation. It is proposed that EDTA-Fe²⁺ complexes catalyze sorbic acid autoxidation, increasing the production of carbonyls, which take part in nonenzymatic browning. Thus, the higher iron content in the EDTA-containing systems might be responsible for the higher rates of sorbic acid degradation as well as for the increase in nonenzymatic browning observed (Campos et al., 1996).

TABLE 3.2
Solubility (%) of Sorbic Acid and Potassium Sorbate

Solvent	Sorbic Acid	Potassium Sorbate
Water, 20°C (pH 3.1)	0.15	58.20
Water, 20°C (pH 4.4)	0.22	—
Water, 20°C (pH 5.9)	1.02	—
Water, 50°C	0.55	61.00
Water, 100°C	4.00	64.00
Corn oil, 20°C	0.08	0.01
Corn oil, 50°C	2.00	0.03
Soybean oil, 20°C	0.52	—
Cottonseed oil, 20°C	1.00	0.01
Sucrose, 10% solution	0.15	58.00
Sucrose, 40% solution	0.10	45.00
Sucrose, 60% solution	0.08	28.00
Sodium chloride, 5% solution	0.11	47.00
Sodium chloride, 10% solution	0.07	34.00
Sodium chloride, 15% solution	0.04	12.00–15.00
Acetic acid, glacial	11.50–12.30	—
Lactic acid, 85.5%	2.25	—
Citric acid, 50%	0.26	—
Phosphoric acid, 85%	0.12	—
Ethanol, 5%	0.16	57.40
Ethanol, 20%	0.29	54.60
Ethanol, 50%	—	45.30
Ethanol, 95%	12.60–14.50	6.50
Ethanol, 100%	12.90–14.80	2.00
Propylene glycol, 20°C	0.02	55.00
Propylene glycol, 50°C	0.05	48.00
Propylene glycol, 100°C	5.50	20.00
Pentane, 25°C	0.15	—
Pentane, 50°C	0.60	—
Pentane, 75°C	1.80	—
Benzene, 25°C	2.34	<0.01
Benzene, 50°C	8.14	—
Benzene, 75°C	24.00	—
Ethyl ether, 20°C	5.00–5.30	0.10
Glycerol, 20°C	0.31	0.20
Acetone, 20°C	9.20	0.10
Methanol, 20°C	12.90	16.00
Cyclohexane, 20°C	0.28	—
Carbon tetrachloride, 20°C	1.30	<0.01

Source: From Sofos (1989).

Losses of sorbic acid during storage of foods depend on sorbate levels, the nature and pH of the food, amino acids, metal ions, light, antioxidants, types of food materials present, moisture content, processing conditions, other additives present, packaging material, and storage temperature and time (Bolin et al., 1980; Vidyasagar and Arya, 1983, 1984; Gerschenson et al., 1986a,b, 1987; Sofos, 1989, 1992, 1994). Sodium chloride at 3.5% and 8.0% enhanced sorbic acid destruction, but at 13.0% it had a protective effect (Guerrero et al., 1990). In general, oxidative degradation of

sorbic acid in foods is less extensive than in aqueous solutions, and it is influenced by the nature of the food and its components and by processing, handling, and storage conditions (Sofos, 1994).

Uptake and diffusion of sorbate into foods should be influenced by properties of the substrate, such as composition, physical state, structure, moisture, and water activity (Giannakopoulos and Guilbert, 1986a,b; Vojdani and Torres, 1990). In general, it is important to be able to predict or to control migration of sorbic acid during processing and storage of foods because it can influence product preservation (Sofos, 1989). When wheat flour doughs are treated with sorbic acid and heated, a significant amount of sorbic acid is not recovered by extraction with methanol. This suggests that sorbic acid is indeed reacting with thiol groups in the flour protein, and the acid-labile nature of this binding is the result of the instability of the thiol-sorbic acid monoadducts under acid conditions (Khandelwal et al., 1992). It is suggested that decomposition of the adducts requires the sulfur atom at position 5 of the substituted 3-hexanoic acid to be protonated and that a proportion of sorbic acid present in flour-based products may be reversibly bound to components of the food (Khandelwal et al., 1992). Jideani and Wedzicha (1994) showed that when sorbic acid is incorporated into millet dough a significant proportion (approximately 40%) of the additive becomes unavailable on extraction with methanol, and a further loss is apparent after the dough is cooked to give the traditional West African food, fura. The lipid and protein fractions that appear to bind sorbic acid or its degradation products were identified with the help of ^{14}C -sorbic acid. Fractionation of the lipids and proteins from fura showed the ^{14}C to be associated with the petroleum spirit crude fat extract ($28\pm 5\%$), the water-soluble ($6.2\pm 0.7\%$), the ethanol-soluble ($4.5\pm 0.7\%$), the salt-soluble ($3.5\pm 0.6\%$), and the acetic acid-soluble components ($0.9\pm 0.3\%$). Dialysis of the ethanol-, salt-, and acetic acid-soluble fractions suggested that ^{14}C activity tends to be associated with the low-molecular-weight components of these fractions. The results show that sorbic acid binds with the lipid components, the water-, salt-, and ethanol-soluble components of fura from pearl millet. A proportion of the binding is with the low-molecular-weight components of these fractions (Jideani and Wedzicha, 1995).

Although the salts of sorbic acid have been commercially developed, broadly tested, and widely used as antimicrobial agents, other derivatives of sorbic acid have also been examined. These include esters, alcohols, aldehydes, amine salts, and amide derivatives. Specifically, some of these compounds are sorboyl palmitate, sorbamide, methyl sorbate, ethyl sorbate, sorbohydroxamic acid, and sorbic anhydride. A mixed anhydride of sorbic and palmitic acids called sorboyl palmitate has found some application in yeast-leavened bakery products. Sorbamide was 1000 times more inhibitory than sorbic acid against yeast alcohol dehydrogenase (Martoadiprawito and Whitaker, 1963); sorbohydroxamic acid, with a dissociation constant (pK_a value) of 8.8, was an effective mold inhibitor over a wide pH range (3.6 to 9.2) (Dudman, 1963; Troller and Olsen, 1976); and sorbic aldehydes were also effective antimicrobials (Troller and Olsen, 1976). Commercial application of such derivatives, however, is hindered by disadvantages, such as low solubility in water, strong off-flavors and odors, and potential health problems (Lück, 1980; Sofos, 1989; Sofos and Busta, 1981).

New controlled-release, water-soluble formulations of sorbic acid in the form of epoxidized polymers of polyvinylpyrrolidone (PVP) containing covalently bonded sorbic acid (polymeric esters of sorbic acid) and complexes of PVP with hydrogen-bonded sorbic acid have been shown to be more effective fungicidal agents than sorbic acid polymeric esters (Tzatzarakis et al., 2000; Charvalos et al., 2001). In addition, these products have low toxicity and are effective in a wide range of concentrations and thus may be considered for food application or feed protection.

Commercial applications of sorbates include use of the free acid or its potassium and, to a lesser extent, calcium or sodium (salts), which are available as powders, granules, suspensions, or solutions. Because the compounds are sensitive to heat, moisture, and light, they are shipped and stored in fiber drums with a moisture barrier in the carton wall. These cartons should be kept closed in cool, dry, and dark storage areas.

Several procedures have been patented for the manufacture of sorbates (Sofos, 1989), but commercial production of sorbic acid has mostly involved the methods of oxidation of

2,4-hexadienal and the condensation reaction of ketene and crotonaldehyde. Numerous patents describing catalysts, reaction conditions, and purification procedures follow the second method, which is most widely used. Alkaline salts of sorbic acid are formed by its neutralization with alkali metal carbonates, hydroxides, and other suitable salts in the presence of necessary additives.

ANTIMICROBIAL ACTIVITY

INHIBITION

Sorbic acid is active against yeasts and molds, as well as against many bacteria (Sofos, 2000). Extensive research during the 1950s demonstrated the impressive effectiveness of sorbates against yeasts and molds and resulted in the extensive use of the compounds as fungistatic agents in many foods. Effective antimicrobial concentrations of sorbates in most foods are in the range of 0.02% to 0.30%.

The inhibitory action of sorbate against yeasts was first documented in the 1950s in fermented vegetable products. The effectiveness of sorbates against yeasts has been documented by numerous studies (Emard and Vaughn, 1952; Ferguson and Powrie, 1957; Geminder, 1959; Pederson et al., 1961; Huang and Armstrong, 1970; El Halouat et al., 1998; Bracey et al., 1998; Piper et al., 1998). Yeasts inhibited by sorbates include species of the genera *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Endomycopsis*, *Hansenula*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Torulasporea*, *Torulopsis*, and *Zygosaccharomyces* (Sofos, 1989). In addition to their effectiveness in fermented vegetables, sorbates inhibit yeasts in fruit juices, wines, cottage cheese, dried fruits, and meat and fish products. Use of sorbates for inhibition of yeasts is especially important in low-pH and/or intermediate water activity (a_w) products, such as carbonated beverages, salad dressings, syrups, tomato products, jams, candy, jellies, and chocolate syrup (Restaino et al., 1982; Liewen and Marth, 1985a). Although, *Zygosaccharomyces rouxii* is considered resistant to sorbate treatments, use of a hurdle approach resulted in a synergistic effect (El Halouat and Debevere, 1996). Hurdles used in combination included a_w , CO₂ atmospheres, and sorbic acid. Under an 80% CO₂ atmosphere, the inhibitory amounts of sorbic acid were reduced by 40% to 50%. Under this atmosphere and at a_w values in the studied range (0.80 to 0.90), potassium sorbate used at 150 ppm resulted in final counts, after 21 days, lower than the inoculum level (10³ CFU/g), whereas no growth was observed when the preservative concentration was increased to 220 ppm (El Halouat and Debevere, 1996).

Numerous studies have also documented the effectiveness of sorbates against molds (Emard and Vaughn, 1952; Deuel et al., 1954a,b; Melnick and Luckmann, 1954a,b; Melnick et al., 1954a,b; Smith and Rollin, 1954a,b; Huang and Armstrong, 1970; Baldock et al., 1979; Kaul et al., 1979; Kivanç, 1992; Garza et al., 1993; Skirdal and Eklund, 1993; Aly, 1996; Fan and Chen, 1999; Matamoros-León, 1999). Mold species inhibited by sorbates belong to the genera *Alternaria*, *Ascochyta*, *Ascospaera*, *Aspergillus*, *Botrytis*, *Cephalosporium*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Cunninghamella*, *Curvularia*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Helminthosporium*, *Heterosporium*, *Humicola*, *Monilia*, *Mucor*, *Penicillium*, *Phoma*, *Pepularia*, *Pestalotiopsis*, *Pullularia*, *Rhizoctonia*, *Rhizopus*, *Rosellinia*, *Sporotrichum*, *Trichoderma*, *Truncatella*, *Ulocladium*, and others. A major application of sorbates in food products is their use for inhibition of molds in cheeses (Chichester and Tanner, 1972). Sorbates also inhibit molds in butter, sausages, fruits and juices, cakes, grains, bread, and smoked fish (Liewen and Marth, 1985a; Sofos, 1989).

Sorbates inhibit the formation of mycotoxins by various molds in culture media and in foods (Bullerman, 1983, 1984, 1985; Liewen and Marth, 1983, 1984; Roland and Beuchat, 1984; Lennox and McElory, 1984; Bhattacharya and Majumdar, 1984; Tsai et al., 1984; Tong and Draughon, 1985; Marshall and Bullerman, 1986; Gourama and Bullerman, 1988). However, neither sorbate nor propionate, applied at 10 mg/ml, were able to depress mycelial weight and aflatoxin production by *Aspergillus flavus* or *Aspergillus parasiticus* cultured in yeast extract-sucrose (YES) broth (Fan

and Chen, 1999). In fact, under certain conditions, subinhibitory levels of sorbate may simulate the production of mycotoxins (Bullerman and Olivigni, 1974; Gareis et al., 1984; Liewen and Marth, 1985a,b; Rusul and Marth, 1987; Monnet et al., 1988; Sanchis et al., 1988). Stimulation of mycotoxin formation by low levels of sorbate depends on species and strains of molds, storage temperature, and other factors (Sofos, 1989).

Bacterial species inhibited by sorbate belong to the genera *Acetobacter*, *Achromobacter*, *Acinetobacter*, *Enterobacter*, *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Arthrobacter*, *Bacillus*, *Campylobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Pediococcus*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Staphylococcus*, *Vibrio*, *Yersinia*, and others (El-Shenawy and Marth, 1988; Zhao et al., 1993; Kouassi and Shelef, 1995a,b; Sofos, 2000; Koodie and Dhople, 2001). Depending on pH and concentration, sorbate inhibited or inactivated *Listeria monocytogenes* in a broth substrate (El-Shenawy and Marth, 1988) and in a cold-pack cheese food (Ryser and Marth, 1988). Potassium sorbate sensitized cells of *L. monocytogenes* and *Zygosaccharomyces bailii* to inactivation by high hydrostatic pressure (Mackey et al., 1995; Palou et al., 1997). In other studies (Kouassi and Shelef, 1995a,b), although sorbate did not affect growth of *L. monocytogenes* in broth, it suppressed cysteine activation of listeriolysin. It was concluded that combinations of sorbate with propionate or lactate, which inhibited growth, could extend shelf life and increase safety (Kouassi and Shelef, 1995a,b; Sofos, 2000). At the relatively high level of 1%, potassium sorbate slightly decreased *L. monocytogenes* presence in two commercial cheese brines and thus could be used as an antilisterial agent in commercial brines, but the cost effectiveness of adding high levels (1%) of substrate is questionable, and their long-term stability in the high-salt and low pH environment of brines is not well documented (Larson et al., 1999). *Staphylococcus aureus* producing staphylococcal thermonuclease (TNase) retained its full activity and was not inhibited even after exposure to chilling and refrigeration temperatures when sorbic acid was applied at 0.04% to 0.5% (Kumar et al., 2000). A hydroxypropyl methylcellulose (HPMC) coating significantly reduced the number of viable *Salmonella* Montevideo cells on the surface of tomatoes; however, the addition of sorbic acid (0.2% to 0.4%) to HPMC did not substantially enhance bactericidal activity (Zhuang et al., 1996). The combination of 0.1% potassium sorbate and 0.1% sodium benzoate substantially decreased *Escherichia coli* O157:H7 contamination and suppressed the growth of yeasts and molds, which could have a protective effect on *E. coli* O157:H7, increasing its survival, and result in spoilage of the product (Zhao et al., 1993). Addition of 0.05% sorbic acid was found to inhibit growth of *E. coli* O157:H7 in apple cider (Koodie and Dhople, 2001). However, it has also been shown that potassium sorbate or sodium benzoate did not affect survival of *E. coli* O157:H7 during storage of apple cider (Miller and Kaspar, 1994). Certain bacterial strains are not inhibited by sorbate, however, and some may even metabolize the compound (Sofos, 1989). Overall, however, sorbates can inhibit Gram-positive and Gram-negative, catalase-positive and catalase-negative, aerobic and anaerobic, and mesophilic and psychrotrophic microorganisms, as well as spoilage and pathogenic bacteria. Inhibition of bacteria by sorbate appears to cause an extension of the lag phase, with a lesser influence on rate and extent of growth (Larocco and Martin, 1981; Chung and Lee, 1981, 1982; Greer, 1982; Zamora and Zaritzky, 1987a,b; Tsay and Chou, 1989). The effect of sorbate on spore-forming bacteria may be exerted on spore germination, outgrowth, and/or vegetative cell division (Sofos et al., 1979a-d, 1980a, 1986; Smoot and Pierson, 1981; Seward et al., 1982; Blocher and Busta, 1985; Lund et al., 1987). Overall, however, sorbic acid is considered as a more effective inhibitor of yeasts and molds than bacteria (Skirdal and Eklund, 1993; Sofos, 1989).

SELECTIVE ACTION

Microbial inhibition by sorbate is variable and depends on species, strains, composition of substrate, pH, a_w , additives present, food-processing treatments, temperature of storage, gas atmosphere, type

of packaging, and concentration of sorbate. Variations and resistance to inhibition by sorbate may lead to failures in preservation and defective food products (Sofos, 1989).

Early studies indicated that sorbate could be used as a selective agent for catalase-negative lactic acid-producing bacteria and clostridia because it was highly inhibitory against catalase-positive organisms (Phillips and Mundt, 1950; Vaughn and Emard, 1951; Emard and Vaughn, 1952; York and Vaughn, 1954, 1955). In contrast, other studies have reported either no effect or inhibition of lactics and clostridia by sorbate (Costilow et al., 1955; Hansen and Appleman, 1955; Hamdan et al., 1971). Overall, however, the inhibitory action against lactics by sorbate is less than that against yeasts, which explains the usefulness of the compound as a preservative in vegetable fermentations. Another bacterium that appears to be more resistant to inhibition by sorbate than other spore formers is *Sporolactobacillus* (Botha and Holzapfel, 1987). Growth of *Gluconobacter oxydans* in the presence of sublethal concentrations of sorbic acid before determination of the minimal inhibitory concentration (MIC) resulted in a substantial increase in the MIC within 1 hour (Eyles and Warth, 1989). In general, various species and strains of microorganisms exhibit different sensitivities to inhibition by sorbate. Varying sensitivities of bacterial species and strains to sorbate may lead to shifts in the microbial flora during storage of foods (Chung and Lee, 1981, 1982; Lahellec et al., 1981; Blocher et al., 1982; Lynch and Potter, 1982; Blocher and Busta, 1983, 1985; McMeekin et al., 1984; Kondaiah et al., 1985).

In addition to bacteria, under certain conditions some species and strains of yeasts and molds are resistant to inhibition by sorbate. Yeast strains resistant to sorbate belong to the genera *Zygosaccharomyces*, *Saccharomyces*, *Torulopsis*, *Brettanomyces*, *Candida*, and *Triganopsis* (Warth, 1977, 1985; Splittstoesser et al., 1978; Restaino et al., 1982, 1983; Bills et al., 1982; Cole et al., 1987; Lenovich et al., 1988; Mihyar et al., 1997). Of 100 yeast strains isolated from spoiled foods and beverages, most tolerated 150 ppm sorbic acid, 40% tolerated 500 ppm, and two strains of *Z. bailii* tolerated 800 ppm of sorbic acid (Neves et al., 1994). Resistance of yeasts to inhibition by sorbate depends on species and strains, sorbate concentration, pH, inoculum level, storage temperature, and previous exposure of the organism to low levels of sorbate (Sofos, 1989). When the yeast cells have been previously adapted to sorbate in media containing glucose or sucrose, subsequent exposure of the cells shows little effect of solute type on sorbate resistance (Lenovich et al., 1988). However, potassium sorbate suppressed growth of *Z. bailii* in salsa mayonnaise more than sodium benzoate (Wind and Restaino, 1995). Potassium sorbate or sodium benzoate resulted in complete inhibition of *Z. rouxii* in high moisture prunes (El Halouat et al., 1998).

Resistance of osmotolerant yeasts to inhibition by sorbate was acquired by preconditioning the yeast to sorbate (Bills et al., 1982). One proposed mechanism of resistance of osmotolerant yeasts has involved an inducible, energy-requiring system that transports the preservative out of the cell (Warth, 1977). Other proposed mechanisms of yeast resistance to sorbate at reduced a_w have been related to yeast cell shrinkage and decreases in membrane pore size, retarding the flow of sorbate into the cell (Restaino et al., 1983), or protection of enzyme systems from inhibition by sorbate through production of compatible solutes, such as polyols (Bills et al., 1982). Exposure of *Saccharomyces cerevisiae* to sorbic acid caused strong induction of two plasma membrane proteins, one of which was identified as adenosine triphosphate (ATP)-binding cassette transporter (Pdr12), which is essential for the adaptation of yeast to growth under weak acid stress and confers weak acid resistance by mediating energy-dependent extrusion of water soluble carboxylate ions (Holyoak et al., 1996; Piper et al., 1998). Exposure of *S. cerevisiae* to 0.9 mM sorbic acid at pH 4.5 resulted in the increased transcription and translation (upregulation) of genes encoding 10 different proteins and the downregulation of three proteins (de Nobel et al., 2001). Functional categories of genes that are induced by sorbic acid stress included cell stress (particularly oxidative stress), transposon function, mating response, and energy generation. The induction of Hsp26, a heat shock protein of *S. cerevisiae*, which occurs during adaptation to sorbic acid, confers resistance to the inhibitory effects of sorbic acid (de Nobel et al., 2001).

DEGRADATION

Animals and certain microorganisms can metabolize sorbate, under certain conditions, as a fatty acid through β -oxidation. When sorbate levels are high, there is also evidence of some ω -oxidation (Deuel et al., 1954b; Lüick, 1980). Like caproic and butyric acids, under normal conditions of alimentation, sorbate is completely oxidized to carbon dioxide and water. Because it is metabolized like other fatty acids, sorbate yields 6.6 kcal/g, of which 50% is biologically usable.

Some mold strains can grow and metabolize sorbate under certain conditions as detected in cheeses and fruit products (Melnick et al., 1954b; Sofos, 1989). Mold strains of the genus *Penicillium* isolated from cheese treated with sorbate were able to grow and metabolize high (0.18% to 1.20%) sorbate levels (Marth et al., 1966; Bullerman, 1977; Finol et al., 1982). It should be noted that 0.1% sorbate is usually sufficient to inhibit sensitive molds (Liewen and Marth, 1985a,b). It appears that selection may occur in sorbate-treated cheeses for certain molds tolerant to the compound (Schroeder and Bullerman, 1985). Products of sorbate metabolism by molds include 1,3-pentadiene, which is a volatile compound formed through a decarboxylation reaction and has a kerosene-like, plastic paint, or hydrocarbon-like odor (Marth et al., 1966; Liewen and Marth, 1985a–c). Other strains of molds that may degrade sorbate belong to the genera *Aspergillus*, *Fusarium*, *Mucor*, and *Geotrichum* (Sofos, 1989). It appears, however, that there is no apparent relationship between sorbate resistance and the toxigenic properties of molds (Tsai et al., 1988). In general, although many molds are sensitive to inhibition by sorbate, certain strains are resistant and can metabolize the compound, using it as a carbon source. Degradation of sorbate by molds depends on species and strains, prior exposure to subinhibitory levels of sorbate, level of inoculum, amount of sorbate present, and type of substrate (Sofos, 1989).

In addition to certain molds, some bacterial strains may also degrade sorbate under appropriate conditions. This metabolism is mostly associated with lactic acid-producing bacterial strains present as high inocula in sublethal concentrations of sorbate (Crowell and Guymon, 1975; Horwood et al., 1981; Liewen and Marth, 1985a). Degradation of sorbate by lactic acid bacteria has been associated with geranium-type off-odors in wines and fermented vegetables, caused by ethyl sorbate, 4-hexenoic acid, 1-ethoxyhexa-2,4-diene, and 2-ethoxyhexa-3,5-diene (Edinger and Splittstoesser, 1986a,b; Sofos, 1989). In general, a geranium-like odor is usually associated with wines treated with sorbate and contaminated with high microbial loads.

INTERACTIONS

The antimicrobial activity of sorbates is influenced by compositional, processing, and environmental factors, such as concentration, other ingredients, pH, a_w , temperature, gas atmosphere, packaging, microbial flora, inoculum size, and other additives (Sofos and Busta, 1981; Sofos, 1989; Steels et al., 2000). These factors can act synergistically or be antagonistic and either enhance or negate the antimicrobial activity of sorbate (Sofos, 1989, 1992).

The MIC of sorbic acid increases with the size of the inoculum; large inocula at high cell concentrations therefore require considerably higher concentrations of the inhibitor to prevent growth than do dilute cell suspensions. A study found a pronounced positive inoculum effect of *Z. bailii* resistance to sorbic acid activity, which was not an artifact caused by insufficient growth time, dehydration of cultures, substantial metabolism of sorbic acid, or binding of sorbic acid to dead cells, but an inoculum effect that may be caused by the diversity of the cells in inocula or initial contamination as regards to sorbic acid resistance (Steels et al., 2000). The study showed that the resistance of *Z. bailii* to sorbic acid was largely the result of the presence of a small fraction of resistant cells and was not heritable or the result of the existence of a mixed culture (Steels et al., 2000). Increasing the concentration of sorbic acid from 200 to 1000 mg/L in apple cider decreased the $D_{50^\circ\text{C}}$ value of *E. coli* O157:H7 from 36 to 5.2 min, about a 7-fold increase in lethality (Splittstoesser et al., 1995). Sorbic acid (0.1%) reduced the $D_{50^\circ\text{C}}$ of *E. coli* O157:H7 during storage

of apple juice from 18 to 5.2 minutes, whereas benzoic acid reduced it to 0.64 min (Splittstoesser et al., 1996).

The antimicrobial action of sorbate is pH dependent and increases as the pH of the substrate decreases, approaching its dissociation constant ($pK_a = 4.76$) (Cowles, 1941; Hoffman et al., 1944; Rahn and Conn, 1944; Lück, 1976, 1980; Sofos and Busta, 1981; Cerruti et al., 1990). Although activity is greater at low pH values, sorbates have the advantage of being effective at pH values as high as 6.5 (Bell et al., 1959, Lück, 1976; Sofos et al., 1979a; Sofos and Busta, 1980, 1981); however, certain studies have indicated antimicrobial activity by sorbate at pH values as high as 7.0 (Raevuori, 1976; Chung and Lee, 1982; Statham and McMeekin, 1988). In contrast, the maximum pH for antimicrobial activity by most other common food preservatives is lower — for example, 5.0 to 5.5 and 4.0 to 4.5 for propionate and benzoate, respectively (Sofos and Busta, 1981).

The increased activity of sorbates at pH values higher than 5.5 is advantageous because it allows for their use in foods of higher pH values in which preservatives, such as parabens, might not be effective owing to their increased solubility in fat. In certain instances, sorbates can partially or totally replace benzoate even in foods of lower pH to avoid possible off-flavors caused by the higher benzoate levels needed for inhibition and to extend the range of microbial groups inhibited compared to benzoate or propionate used singly (Melnick et al., 1954a; Gooding et al., 1955; Sofos and Busta, 1981, 1982, 1993).

The increased antimicrobial activity of sorbate at lower pH values has been attributed to the increased amount of undissociated acid present, which is believed to be the effective antimicrobial form (Lück, 1980; Sofos and Busta, 1981; Lund et al., 1987; Sofos, 1989; Skirdal and Eklund, 1993). This popular theory has been questioned, however (Sofos, 1989). Studies have indicated that the dissociated sorbic acid also had antimicrobial activity, but it was 10 to 600 times less inhibitory than the dissociated acid (Eklund, 1983; Statham and McMeekin, 1988). In environments of pH higher than 6.0, however, more than 50% of the inhibition was the result of dissociated sorbic acid (Eklund, 1983). Nevertheless, it is believed that both undissociated and dissociated sorbic acids have antimicrobial activity (Skirdal and Eklund, 1993). Use of artificial saltwater prevented dissociation of the 1% sorbic acid, which exhibited favorable antimicrobial properties against *Vibrio vulnificus*, supporting previous evidence that sorbic acid is effective in its undissociated form (Sun and Oliver, 1994). Sorbic acid (0.1%) enhanced destruction of *E. coli* O157:H7 cells at pH 3.4 but not at pH 6.4 (Liu et al., 1997). Increased amounts of fat in a product reduce the concentration of sorbate in the water phase, where it is needed for microbial control (Oka, 1960). Other food ingredients (e.g., salt and sugars) also reduce the concentration of sorbate in the aqueous phase (Gooding et al., 1955; Liewen and Marth, 1985a). Sugar and salt, however, act synergistically to enhance the antimicrobial activity of sorbate (Costilow et al., 1955, 1956, 1957; Sheneman and Costilow, 1955; Acott et al., 1976; Robach and Stateler, 1980; Beuchat, 1981c). In general, solutes should increase the inhibitory activity of sorbate by reducing the a_w of the substrate (Sofos, 1989; Cerruti et al., 1990). Sucrose, glucose, and sodium chloride, however, have reduced the synergistic effect of sorbate and heat on thermal inactivation of microorganisms (Beuchat, 1981a–c; Cerruti et al., 1988). Sodium chloride (1.25 and 2.5%) reduced the inhibition of *Clostridium botulinum* by sorbate in a nutrient broth (Wagner and Busta, 1984, 1985a,b). Preconditioning of *Saccharomyces rouxii* cells in 60% sucrose + 0.1% sorbate rendered the cells more sensitive to inhibition by sorbate than preconditioning in 0% sucrose + 0.1% sorbate (Bills et al., 1982). Lowering the a_w enhanced the resistance of the same organism to increasing concentrations of sorbate (Restaino et al., 1981, 1983). Not only are certain strains of microorganisms resistant to inhibition by sorbate, but increased levels of microbial contamination reduce antimicrobial activity. Thus, sorbates should be used to preserve foods processed using good manufacturing practices, not as a substitute for appropriate sanitation and hygienic practices.

Interactions of sorbate with heat may affect the rate and extent of microbial destruction during heating, as well as dormancy and recovery of heated microorganisms (Sofos, 1989). Sorbate may enhance heat activation and destruction of spores, and it may inhibit the repair and growth of

thermally injured organisms (Beuchat, 1980, 1981a–d, 1982; Lusher et al., 1984; Banks et al., 1988; Lopez et al., 1996; Oloyede et al., 1994; Splittstoesser et al., 1995), but the effect of sorbate on thermal inactivation and recovery of injured microorganisms is variable among species and strains. Low concentrations of sorbic and fumaric acids in the heating medium had little effect on the heat resistance of *Eurotium herbariorum*, a true aerophilic mold involved in the spoilage of grape preserves (Splittstoesser et al., 1989). Concentrations of sorbate as high as 0.1% had little effect on the thermal resistance of ascospores of *Neosartorya fischeri*, but growth of surviving spores that had been exposed to high temperatures was greatly inhibited by sorbate concentrations as low as 0.007% (Splittstoesser and Churey, 1989). Another report also indicated that potassium sorbate inhibited the heat-resistant *N. fischeri* (Nielsen et al., 1989). Sorbate may also eliminate the protective effect of sucrose against the thermal inactivation of yeasts and molds (Sofos, 1989). Sorbic and benzoic acids affected the thermotolerance and heat shock response of *S. cerevisiae* depending on pH (Cheng and Piper, 1994; Sofos, 2000). At low pH, sorbate inhibited induction of thermotolerance by sublethal heat shock, but at pH 5.5 it acted as a powerful inducer of thermotolerance in the absence of sublethal heat treatment. Sorbic acid was found to induce thermotolerance without inducing the heat shock response through accumulation of trehalose in *S. cerevisiae* (Cheng et al., 1999).

It is of particular interest that 0.1% potassium sorbate did not have any significant effects on the heat resistance of *Bacillus stearothermophilus* spores in distilled water, and as such would therefore be important only in improving microbiological stability through inhibitory effects on germination and/or outgrowth of heat-damaged spores (López et al., 1996). Tolerance of *Z. rouxii* to sorbate was enhanced in cells preconditioned to elevated sorbate concentrations, especially in media containing 0.1% sorbate. Cells grown in sucrose-supplemented media tolerated higher concentrations of sorbate than did those grown in glucose-supplemented media. Heat resistance increased substantially when cells were grown in media containing sorbate, and particularly in the presence of sucrose as opposed to glucose. The finding that plasmolysis increased in cells grown in sucrose- as opposed to glucose-containing media provides evidence that cell morphology influences heat resistance (Golden and Beuchat, 1992).

Inhibition of microbial growth by sorbate is more effective as storage temperature decreases, indicating that the compound should be more useful as a preservative in refrigerated foods (Pederson et al., 1961; Park et al., 1970; Park and Marth, 1972; Robach, 1980; Roberts et al., 1982; Roland and Beuchat, 1984; Roland et al., 1984; McMeekin et al., 1984; Tuncan and Martin, 1985). Combinations of acidification and treatment with sorbate may enhance the storage stability of fruit juices even at temperatures higher than normal refrigeration (5°C) (Alli and Kermasha, 1989). Reductions of 5 to 10 log-units of *E. coli* O157:H7 or *Salmonella typhimurium* DT104 in apple cider were achieved through freeze-thaw treatments in the presence of sorbic acid (Uljas and Ingham, 1999).

The antimicrobial activity of sorbate is usually enhanced under vacuum or modified gas atmosphere storage conditions, as indicated with several food items, including meat (Wagner et al., 1982; Myers et al., 1983; McMeekin et al., 1984) and fish products (Bremmer and Statham, 1983; Statham et al., 1985). Combinations of carbon dioxide and sorbate have also been reported as effective inhibitors of microbial growth (Danzinger et al., 1973; Elliot and Gray, 1981; Elliot et al., 1982, 1985; Gray et al., 1984). Sorbic acid (0.2% to 0.4%), however, did not enhance the bactericidal activity of cellulose-based edible films against *Salmonella* Montevideo (Zhuang et al., 1996).

Although food acids may reduce the water solubility of sorbate, they can enhance its antimicrobial activity by increasing the concentration of undissociated sorbic acid. In addition, the specific anion itself may contribute antimicrobial activity (Juven, 1976; Huhtanen and Feinberg, 1980; Huhtanen et al., 1981, 1983). Specific effects, however, vary with substrates, microorganisms, and types of acids (Restaino et al., 1981, 1982; Sofos, 1989).

Combinations of sorbate with antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG), had increased antimicrobial activity compared with individual components (Klindworth et al., 1979; Davidson

et al., 1981; Lahellec et al., 1981; Morad et al., 1982; Poerschke and Cunningham, 1985). Variations existed, however, with types of microorganisms, antioxidants, and substrates. These combinations offer the advantage of simultaneous inhibition of microbial growth and development of rancidity. Potassium sorbate was found more inhibitory against yeasts than hydroxycinnamic acid (Stead, 1995).

Several studies have also indicated increased antimicrobial effects when sorbate was combined with various phosphates (Ivey and Robach, 1978; Robach, 1979b; Seward et al., 1982; Nelson et al., 1983; Wagner and Busta, 1983; Sofos, 1986a; Thomas and Wagner, 1987; Mendonca et al., 1989). Combinations of sorbate with benzoate or propionate may be used to expand the range of microorganisms inhibited with reduced concentrations of each preservative.

The synergistic effect observed when vanillin and potassium sorbate were used in combination could be considered for use to reduce the amounts needed to inhibit mold growth (Matamoras-León et al., 1999). *Penicillium glabrum* was considered as the most resistant *Penicillium* mold tested and was found to be inhibited by the combination of 500 ppm of vanillin and 300 ppm of potassium sorbate for at least 1 month at 25°C, pH 3.5, and a_w 0.98; these concentrations of chemicals represented a greater than 50% reduction of the amounts needed to suppress growth when used individually (Matamoras-León et al., 1999). In addition, mixtures of sorbate with various antibiotics have demonstrated increased antimicrobial activity (Schmidt, 1960; Amano et al., 1968; Miller and Brown, 1984; Gourama and Bullerman, 1988). Interactions and increased antimicrobial effects have also been observed in combinations of sorbates with propionate, ascorbate, certain amino acids, fatty acids, sucrose fatty acid esters, sulfur dioxide, propylene glycol, glucose oxidase, and other compounds (Ferguson and Powrie, 1957; Ough and Ingraham, 1960; Robach et al., 1981; Kabara, 1984; Kadam et al., 1985; Marshall and Bullerman, 1986; Bell and De Lacy, 1987; Tellez-Giron et al., 1988).

Numerous publications and patents describe interactions of sorbate with many other factors, as well as multifactorial interactions (Sofos, 1989; Thomas et al., 1993; Guerrero et al., 1994; Deak and Beuchat, 1994). The microbial stability of shelf-stable banana puree was enhanced through inhibition of inoculated osmophilic and nonosmophilic yeasts, molds, *Bacillus coagulans*, *Clostridium pasteurianum*, and *Clostridium butyricum* by adjustment of a_w to 0.97 and pH to 3.4 and addition of 250 ppm ascorbic acid, 100 ppm potassium sorbate, 400 ppm sodium bisulfite, and mild heat (Guerrero et al., 1994). The effect of temperature, pH, sodium chloride concentration, and potassium sorbate was evaluated against growth of three foodborne bacterial pathogens (*Bacillus cereus*, verocytotoxigenic *Escherichia coli*, and *S. aureus*) using gradient gel plates (Thomas et al., 1993). Sorbate was completely effective against *E. coli* at all temperature/pH/NaCl combinations and was the most effective preservative tested against *B. cereus*. Increase in the acidity and/or the NaCl concentration improved the effect of all the preservatives, except nitrite when used against *S. aureus*. At <25°C, sorbate was more effective than benzoate against *S. aureus* when used with higher concentrations of NaCl (Thomas et al., 1993). As indicated, *Z. bailii* is highly resistant to individual inhibitory factors, including sorbate. A study found that growth was not inhibited at pH 3.8, in a medium containing 0.06% potassium sorbate, at a_w of 0.93, or at 10°C (Deak and Beuchat, 1994). However, interactions between two or more of these factors resulted in marked inhibition with the inhibitory effect of potassium sorbate occurring at the highest concentration (0.06%) combined with a_w values lower than 0.95 (Deak and Beuchat, 1994). The ability of *C. botulinum* nonproteolytic type B to grow in the presence of sorbic acid was at least as great at 20°C as at 30°C (Lund et al., 1990). After 14 days at 30°C in the presence of 280 mg undissociated sorbic acid/L, the log probability of growth of a single vegetative cell is approximately -4. At temperatures of 12°C and below, sorbic acid resulted in marked inhibition; the log probability of growth of a single bacterium at 12°C after 60 d at pH 5.5 in the absence acid being 0 and -1 under the same conditions but in the presence of a calculated undissociated sorbic acid concentration of 200 mg/L, equivalent to a total sorbic acid concentration of 1300 mg/L, the probability of growth was approximately -6 (Lund et al., 1990).

TABLE 3.3
Common Applications of Sorbates as Antimicrobial Agents

Products	Levels (%)
Dairy products: aged cheeses, processed cheeses, cottage cheese, cheese spreads, cheese dips, sour cream, yogurt	0.05–0.03
Bakery products: cakes, cake mixes, pies, fillings, mixes, icings, fudges, toppings, doughnuts	0.03–0.30
Vegetable products: fermented, pickles, olives, relishes, fresh salads	0.02–0.20
Fruit products: dried fruit, jams, jellies, juices, fruit salads, syrups, purees, concentrates	0.02–0.25
Beverages: still wines, carbonated and noncarbonated beverages, fruit drinks, low-calorie drinks	0.02–0.10
Food emulsions: mayonnaise, margarine, salad dressings	0.05–0.10
Meat and fish products: smoked and salted fish, dry sausages	0.05–0.30
Miscellaneous: dry sausage casings, semimoist pet foods, confectionary	0.05–0.30

Source: From Sofos (1989).

In general, the extent of the antimicrobial activity of sorbate under commercial conditions is difficult to predict because it is influenced by a variety of factors. These factors include product pH and composition (fat and moisture), presence of other ingredients and preservatives, product processing, sanitation and contamination, packaging, gas atmosphere, and storage temperature (Sofos and Busta, 1981; Sofos, 1989).

APPLICATIONS

Sorbates are widely used food preservatives throughout the world. The most commonly used forms include sorbic acid and the potassium salt. The compounds find application in human foods of all types, animal feeds, pharmaceuticals, cosmetic products, packaging materials, and technical preparations that come in contact with foods or the human body. In the United States, sorbates may be used in any food product that allows generally recognized as safe (GRAS) food additives and in approximately 80 additional food products that have federal standards of identity (Sofos, 1989, 1992).

As yeast and mold inhibitors, sorbates have found wide application in various foods, including dairy products, bakery items, fruit and vegetable products, edible fat emulsion products, certain meat and fish products, and sugar and confectionery items (Sofos, 1989) (Table 3.3). However, as is the case with all food preservatives, sorbates should not be considered as substitutes for good sanitation or agents to be used to improve the quality of partially spoiled or degraded foods. They should be considered as adjuncts to proper sanitation and hygiene.

Application methods for sorbates include direct addition in the formulation, spraying or immersing the food material in a solution, dusting with a powder, or addition in a coating or packaging material (Table 3.4). Selection of the most appropriate method depends on processing procedures, type of food, objectives to be accomplished, equipment available, and convenience (Sofos, 1989). Amounts of sorbate used in foods are in the range of 0.02% to 0.3%. Although these concentrations have no major impact on food quality, higher levels may cause undesirable changes in the taste of most foods. In general, amounts of 0.1% to 0.3% are tolerated, but levels as low as 0.1% may be detectable in some foods.

DAIRY PRODUCTS

Cheese preservation is one of the most common applications of sorbates. In the United States, federal standards of identity permit the use of sorbates as preservatives in more than 40 types of cheese, cheese spreads, and cheese food [21 C.F.R. Part 133 (2001)].

TABLE 3.4
Methods of Application of Sorbates

Methods	Products	Solution (%)	Application Level (%)
Immersion	Dairy: provolone, Siciliano, Caciocarallo, pasta filata, Edam, Muenster, Gouda	20–40	0.1–0.3
	Dry sausage or casings	2.5–10	0.05–0.3
Spraying	Dairy: Swiss, Gruyere, Emmentaler	20–40	0.1–0.3
Immersion or spraying	Dairy: cheddar, Colby, Monterey Jack, blue, mozzarella	20–40	0.01–0.3
	Dried fruits: Prunes	2.5–5	0.02–0.06
	Raisins	5	0.25
	Figs	2.5–5	0.05–0.1
Dry mixing	Smoked and salted fish	5	0.05–0.15
	Bakery: Cheesecake		0.1–0.3
	Angel food cake		0.03–0.06
	Fruitcake		0.1–0.4
	Cake mixes		0.05–0.1
	Doughnut mixes		0.05–0.1
Direct addition	Pie crust dough		0.05–0.1
	Dairy: processed cheese, spreads		0.1–0.2
	Cottage cheese		0.05–0.075
	Bakery: icings, fillings, toppings, fudges		0.05–0.1
	Vegetables: fresh salads (potato, vegetable, slaw, pickles, relishes, olives, sauerkraut)		0.05–0.15
	Fruits: syrups, beverages, drinks		0.025–0.075
	Still wines		0.02–0.04
	Other: Margarine		0.05–0.1
Dry mixing or direct addition	Semimoist pet food		0.1–0.3
	Bakery: pound cake		0.075–0.15
	Devil's food cake		0.3
	Chocolate cake		0.1–0.3

Source: From Sofos (1989).

Kivanç (1992) found that *Penicillium* was the major genus found on cheddar cheese, Swiss cheese, and domestic and imported cheeses. Sorbates are widely used in preserving cheese owing to the susceptibility of all types of cheese to microbial deterioration, especially surface mold growth during storage, aging, and distribution; the high activity of sorbates against molds; and the improved action of sorbates at high pH values compared to other common preservatives (Lück, 1976, 1980; Sofos and Busta, 1981). In these applications sorbates not only prevent cheese spoilage by mold, but they also protect human health by preventing the formation of toxic mold metabolites (mycotoxins). It is obvious, however, that sorbates should not be used in mold-ripened cheeses, such as Roquefort or blue cheese and Camembert cheese, in which they can interfere with the desirable molds involved in the manufacture of these cheeses.

Sorbate application, in general, is limited to the surface of cheese, where mold development usually occurs. In certain products, however (e.g., cottage cheese), sorbic acid may be incorporated into the cheese curd. The method of application varies with the type of cheese; preservation objectives; available facilities and equipment; and regulations, which may vary among countries (Lück, 1976, 1980). Addition of sorbate does not significantly affect the organoleptic properties of mozzarella cheese; however, depending on the technique used to treat the cheese (i.e., dipping or in brine salting), there may develop a slight objectionable flavor (Aly, 1996). For application on

the surface of the cheese, one of the following procedures may be followed: (1) potassium sorbate in solution or in a brine, which can be used to dip, spray, or wash; (2) a sorbic acid powder for dusting; and (3) sorbic acid, potassium, or calcium sorbate impregnated into the packaging material or other coatings (e.g., wax) used in cheese storage and marketing (Han et al., 1998; Sofos, 1989; Weng and Chen, 1997). Treatment of mozzarella cheese with potassium sorbate in the kneading water or brine may be more effective than dipping (Aly, 1996). Amounts of sorbic acid permitted and applied in cheese preservation range from 0.05% to 0.30% (Lück, 1980; Sofos and Busta, 1981). Levels of 0.05% to 0.07% sorbic acid are generally used in direct addition to cheese. Concentrations applied to cheese surfaces range from 0.1 to 0.3 g/dm², whereas when applied to packaging films, amounts of 2 to 4 g/m² are used (Lück, 1980). The appearance of *Penicillium verrucosum* var. *cyclopium* mycelia and spores isolated from cheeses and inoculated as suspensions into YES broth containing 1000 µg of potassium sorbate/ml was not detected at 5°C, 15°C, and 25°C after 30 days (Kivanç, 1992). Addition of potassium sorbate (≤6%) to mozzarella cheese inhibited growth of *Streptococcus salivarius* var. *thermophilus* and *Lactobacillus delbrueckii* var. *bulgaricus*, and was especially effective against mold and yeast contamination by *Penicillium roqueforti* and *Mucor miehi*, respectively (Aly, 1996). Inclusion of sorbic acid in cottage cheese was effective as a preservative but not against *L. monocytogenes* (Piccinin and Shelef, 1995); however, at the relatively high level of 1%, potassium sorbate inhibited *L. monocytogenes* survival in two commercial cheese brines (Larson et al., 1999). In another study, potassium sorbate (0.3%) or sodium benzoate (0.3%) showed a significant effect in delaying growth of *E. coli* O157:H7 in queso fresco, soft cheese (Kasrazadeh and Genigeorgis, 1995). Milk acidification to pH 6.0 with hydrochloric, acetic, or propionic acid significantly increased the effectiveness of potassium sorbate, especially at lower (≤6.6°C) temperatures (Kasrazadeh and Genigeorgis, 1994). Growth of *Salmonella* in soft cheese was inhibited if the cheese was prepared from milk that was acidified with propionic acid to pH 5.9, potassium sorbate was added to the cheese (pH 6.0) at levels of ≥0.3%, and the cheese was stored at ≤30°C (Kasrazadeh and Genigeorgis, 1994).

Absorption of the compound from the surface into the cheese is dependent on the porosity of the surface and the amount and distribution of fat in the cheese. Calcium sorbate may be preferred in cheese preservation over other forms of sorbate owing to its solubility properties. The compound is insoluble in fat and only slightly soluble in water. Therefore, when applied to the surface of hard, ripened cheeses, most of the preservative remains on the surface of the cheese without migrating inside the cheese or dissolving in the water on the shelves during the long maturation period of these cheeses (Lück, 1976). In addition to reducing cheese spoilage and the possibility of aflatoxin formation, use of sorbates in aged cheeses also reduces labor and other costs through reduction of the need for cheese washing and trimming. Furthermore, addition of potassium sorbate may increase the meltability and improve the fat leakage of certain cheeses (Aly, 1996).

In labaneh (a yogurt product) produced under strict hygienic processing measures and effectively chilled, initial numbers of yeasts were low and relatively low concentrations of potassium sorbate and sodium benzoate (<150 and <250 mg/kg, respectively) extended the shelf life by 14 days. Labaneh with higher initial yeast counts needed higher concentrations of preservative (>300 and >400 mg/kg for K-sorbate and Na-benzoate, respectively) to maintain stability when kept at 5°C for 7 or 14 days. These concentrations are in agreement with use of these preservatives in other dairy products, such as cottage cheese and yogurt (Mihyar et al., 1997).

VEGETABLE PRODUCTS

The water-soluble salts of sorbic acid are used widely in the preservation of a variety of plant products, including fresh, fermented, and pickled vegetables (Sofos, 1989). The use of sorbates in the processing of vegetables was one of the first applications, and it was initiated through extensive research performed during the early stages of sorbate testing. This research demonstrated the effectiveness of sorbates against undesirable organisms without interfering with the desirable

fermentations. Use of sorbates at levels between 0.05% and 0.20% inhibits growth of organisms not desired in vegetable fermentations, such as yeasts, molds, and putrefactive bacteria. At these concentrations the selective action of sorbic acid allows growth of the desirable lactic acid-producing organisms. Selection of precise levels of sorbate, however, depends on products and specific fermentation conditions (e.g., salt concentration). Inclusion of sorbate at the initial stage of the process results in a “clean” fermentation and prevents turbidity development in the final product (Jones and Harper, 1952; Costilow et al., 1955; Lück, 1980). Mold and yeast spoilage is also prevented by sorbates in pickled vegetable products (e.g., cucumbers and olives). Addition of 0.02% to 0.05% sorbate to a 15% to 20% brine retards yeast scum formation. Levels of 0.02% to 0.10% sorbate reduce the number of bloaters and poor seed cavities. In sweet cucumber packs, 0.1% sorbate and 0.5% acetic acid completely inhibit microbial spoilage at sucrose concentrations ranging from 2% to 40%. In sweet relishes, sorbate is more effective than benzoate owing to the pH of these products. Also, the pH values of fermented green olives favor the use of 0.02% to 0.05% sorbates in the fermenting and holding tanks. Sorbate levels in the range of 0.05% to 0.10% may be used to preserve refrigerated fresh salads. Other vegetable products preserved with sorbates include tomato products and fermented Asian sauces (Chichester and Tanner, 1972; Lück, 1980; Sofos, 1989).

FRUIT PRODUCTS

Fruit products preserved with sorbates throughout the world include dried fruits, fruit juices and syrups, fruit cocktails, jams, jellies, preserves, beverages, and wines. Sorbate concentrations as low as 0.02% to 0.05% are adequate for preserving high-moisture dried fruits (e.g., prunes, raisins, figs). The higher moisture content in these products is preferred by the consumers, but it makes them susceptible to mold and yeast spoilage. Depending on the particular product, sorbate acts as an inhibitor of yeasts, molds, or bacteria. The lower the moisture content of the product (e.g., dried fruits), the lower is the sorbate level needed for preservation. Also, in high-sugar products (e.g., jams and jellies), smaller quantities of sorbic acid are adequate for preservation because of the synergistic action of sorbate with sugar (Lück, 1980). In these products, sorbate is either added directly into the product or applied to the surface of the product or packaging material. Potassium sorbate was found more effective than chitosan in inhibiting growth of *Aspergillus niger* on low-sugar candied “kumquat” (Fang et al., 1994). Other fruit products that may be preserved with sorbates include maraschino cherries and strawberry puree.

In fruit juice and drink processing, sorbates are used mostly at the preprocessing stage, together with sulfur dioxide and pasteurization, to inhibit chemical, enzymatic, or microbiological deterioration (e.g., fermentation). Concentrations as low as 0.02% to 0.10% are adequate in improving the preservation of soft drinks. Combinations of sorbic acid and sulfur dioxide are also used in the preservation of high-pulp fruit juices. In these products sorbic acid acts as the microbial inhibitor and sulfur dioxide prevents oxidation and enzymatic spoilage (Lück, 1980). Potassium sorbate and sodium benzoate were highly effective in reducing yeast and mold populations in tomato juice (Bizri and Wahem, 1994). Use of the combination of 0.1% sodium benzoate and 0.1% potassium sorbate substantially inhibited *E. coli* O157:H7 in apple cider, while additionally suppressing the growth of yeasts and molds, which may influence the survival of *E. coli* O157:H7 and result in spoilage of the product (Zhao et al., 1993). Another study showed that *E. coli* O157:H7 growth was inhibited in both tryptic soy broth and in apple cider by sorbic acid applied at 0.05% and as such may be an option that processors have to reduce contamination in their products (Koodie and Dhople, 2001).

In wine processing, sorbates are used to prevent refermentation (Parish and Carroll, 1988a,b). Potassium sorbate is preferred because of its solubility. Because potassium may result in potassium bitartrate precipitation in some wines, however, sodium sorbate may be used to avoid the problem. Good wine preservation is achieved with concentrations of 0.02% sorbic acid in combination with

0.002% to 0.004% sulfur dioxide. Sorbate acts as the inhibitor of yeast refermentation during storage, and sulfur dioxide protects against enzymatic and oxidative changes and bacterial fermentation. Sorbate levels above 0.03% are not recommended in wine preservation because they may impart an off-flavor to the delicate taste of wine. Sorbate can also be added before bottling the wine and is very valuable in the preservation of sweet wines. It has also been used in champagne preservation (Sofos, 1989).

Sorbates, in general, may be more suitable for use in fruit products than other common preservatives because of their milder organoleptic properties and their neutral taste, which is especially important in preservation of juices, soft drinks, and other beverages. Often, however, sorbates are used in combination with benzoate in preserving fruit juices and drinks. In fruit preserves (e.g., jams, jellies, and marmalades), mold inhibition may be achieved by sprinkling sorbate on the surface of the product. In dried products with an irregular surface conformation (e.g., figs), the dipping method of application is more effective (Sofos, 1989).

BAKERY PRODUCTS

Although propionic acid-based preservatives are the most widely used compounds in preserving bakery goods, sorbates are also valuable because they are active at higher pH values and effective against molds (Chichester and Tanner, 1972; Lück, 1980). In addition to preventing mold spoilage, sorbates may also be useful in preventing aflatoxin formation (Lück, 1980). Concentrations of 0.03% to 0.30% sorbic acid are adequate for preserving bakery products. Sorbic acid and calcium propionate have been shown to be significantly more effective preservatives than salt and sugar when used to prolong the shelf life of bread (Doulia et al., 2000). In addition to undesirable molds, sorbate also inhibits rope-forming bacteria and pathogens, such as *S. aureus*, in cream pies (Schmidt et al., 1969; Sofos, 1989). Potassium sorbate increased the shelf life of acidified tortillas, especially when used in combination with calcium propionate (Tellez-Giron et al., 1988).

Use of sorbates must be avoided in products raised by yeasts, unless the amount of yeast and the leavening time are increased. Yeast-leavened products may be protected with sorbate spraying after baking. In such applications sorbate may be used in the coating oil or the pan grease (e.g., brown-and-serve products). To avoid the effect of sorbates on desirable yeasts in yeast-leavened products, the derivative sorboyl palmitate, which is a mixed anhydride of sorbic and palmitic acids that has no direct antimicrobial activity, may also be used; upon heating in the baking process, the compound is chemically degraded and releases sorbic acid after the yeast-leavened dough is raised. Use of this compound has the advantage of preserving yeast-leavened bread without interfering with the fermentation process (Lück, 1976, 1980).

Sorbates should be valuable in baking powder-raised products, including cakes and confectionery. Concentrations of 0.05% to 0.10% sorbate inhibit the spoilage of cake icings, toppings, and fillings. Sorbates are also used in the preservation of pie crusts and fillings, refrigerated dough products, pizza shells and toppings, muffins, and doughnut mixes (Sofos, 1989, 1999).

MEAT PRODUCTS

The only approved use of sorbates in meat products in the United States is to suppress mold growth on the surface of dry sausages during the drying period. For this purpose a solution of up to 10% potassium sorbate may be used to protect unrefrigerated dry sausages. However, sorbates are more common meat preservatives in other countries, such as Japan and Korea (Sofos, 1989).

In 1974, Tompkin et al., considering the permitted use of potassium sorbate as a mold inhibitor in dry sausages and reports suggesting that sorbic acid was promoting growth of clostridia (Emard and Vaughn, 1952; York and Vaughn, 1954), examined its activity against various pathogenic bacteria in an uncured, cooked sausage product. A few sporadic studies in the 1950s and 1960s also indicated its potential as a preservative for meat products (Sofos, 1989). Tompkin et al. (1974)

showed that sorbate retarded growth of *Salmonella* species and *S. aureus* and delayed growth and toxin production by *C. botulinum*. This work (Tompkin et al., 1974) was followed by extensive testing that examined the action of sorbates against clostridia and other pathogens in a variety of fresh, uncured, and processed cured meat products (Sofos, 1981, 1989; Robach and Sofos, 1982; National Academy of Sciences 1982). The reason for extensive testing of the antimicrobial properties of sorbate in meat products was the perceived need for an alternative to sodium nitrite as an inhibitor of *C. botulinum* in cured meat products. Nitrite was reported to be a potential carcinogen, and it can react with amines to form carcinogenic nitrosamines during meat product processing and cooking (Sofos et al., 1979a; National Academy of Sciences, 1981).

Extensive studies published in the 1970s and the 1980s established the antibotulinal and overall antimicrobial activity of sorbates in various cured and uncured meat and poultry products. In addition, the effect of sorbates on overall product shelf life, eating quality, nitrosamine formation, and safety were examined. These studies have been thoroughly reviewed by Sofos and Busta (1980), Sofos et al. (1979a), Robach and Sofos (1982), Sofos (1981, 1989), and the National Academy of Sciences (1981, 1982). The antimicrobial activity of sorbate was demonstrated in bacon (Ivey et al., 1978; U.S. Department of Agriculture, 1979a,b; Sofos et al., 1980b), comminuted pork products (Ivey and Robach, 1978; Robach et al., 1978; Robach, 1979b), beef and pork frankfurters (Sofos et al., 1979b; Wagner and Busta, 1983), pork slurries (Roberts et al., 1982; Draughon et al., 1982), uncured cooked sausage (Tompkin et al., 1974), sliced bologna (Chang et al., 1983), raw (Mendonca et al., 1989) and cooked pork chops (Prabhu et al., 1988), beef steaks (Unda et al., 1990), poultry products (Robach et al., 1980c; Sofos et al., 1979b, 1980a; Huhtanen and Feinberg, 1980; Hall and Maurer, 1980; Nelson et al., 1983), and hamburger beef (Yamamura et al., 2000). In addition to *C. botulinum*, other pathogenic and spoilage bacteria inhibited by sorbate in various meat products include *S. aureus*, *Salmonella*, *Clostridium perfringens*, *Escherichia coli*, *Yersinia enterocolitica*, *Brochothrix thermosphacta*, *Serratia liquefaciens*, *Lactobacillus*, *Clostridium sporogenes*, *Bacillus cereus*, *Bacillus licheniformis*, *Pseudomonas*, mesophiles, psychrotrophs, and lipolytic organisms (Kaloyereas et al., 1961; Perry et al., 1964; Tompkin et al., 1974; Robach and Ivey, 1978; Cunningham, 1979; Pierson et al., 1979; Petaja et al., 1979; Robach, 1979a; Sofos et al., 1980b; To and Robach, 1980a,b; Hallerbach and Potter, 1981; Wagner et al., 1982; Morad et al., 1982; Rice and Pierson, 1982; Greer, 1982; Bushway et al., 1982; Chang et al., 1983; Myers et al., 1983; Gray et al., 1984; McMeekin et al., 1984; Elliot et al., 1985; Sofos, 1986b,c; Zamora and Zaritzky, 1987a,b). Products in which these microorganisms are inhibited include bacon and other cured meats, cured poultry products, raw beef and pork, and uncured poultry meat and carcasses. The antimicrobial activity of sorbate in these products has been enhanced when combined with nitrite, sodium chloride, phosphates, antioxidants, acids, low pH, low storage temperature, low oxygen, and increased carbon dioxide atmospheres (Sofos, 1989).

Sorbate in meat products (<0.3%) had no major adverse effects on sensory qualities, such as color and flavor (Frank, 1977; Ivey et al., 1978; Bauermann, 1979; Kemp et al., 1979, 1983; Paquette et al., 1980; Amundson et al., 1981a,b; Berry and Blumer, 1981; Berry et al., 1981; Chambers et al., 1981; Huhtanen et al., 1981, 1983; Wagner and Gehrke, 1982; Vareltzis et al., 1984; Vareltzis and Buck, 1984). In addition, inclusion of sorbate and reduction of nitrite levels in bacon-pumping brine solutions resulted in reduced levels of nitrosamines in the fried product (Ivey et al., 1978; U.S. Department of Agriculture, 1979b; Robach et al., 1980b). Based on the results of the various studies, sorbate was proposed as a means of reducing nitrite and nitrosamine levels in bacon while maintaining antimicrobial activity and inhibition of *C. botulinum* spores during temperature abuse of the product. This proposal, however, did not take effect following a report that consumption of experimental bacon resulted in allergic-type reactions in certain individuals (Berry and Blumer, 1981). It should be noted, however, that no other studies reported undesirable flavors or allergic reactions (Sofos, 1989) and that a National Academy of Sciences committee concluded that although the flavor of bacon treated with combinations of sorbate and nitrite was not the same as the flavor

of bacon treated only with nitrite, the flavors of both types of bacon were equally desirable (National Academy of Sciences, 1982).

MISCELLANEOUS FOOD PRODUCTS

Sorbates are commonly used in the preservation of oil-in-water or water-in-oil emulsion-based products, such as margarine, mayonnaise, salad dressings, delicatessen items, and similar products. In certain instances and for better preservation, sorbic acid may be mixed with its potassium salt or with other preservatives, such as benzoates. Other food-related applications of sorbates include sugar-based and confectionery products at concentrations of 0.05% to 0.20%, at which they prevent growth of molds and osmophilic yeasts in items such as fillings for chocolate and pralines (Chichester and Tanner, 1972; Lück, 1976, 1980; Sofos and Busta, 1981). Sorbates are also used to prevent mold growth on the surface of dried and smoked fish (e.g., dried cod). In Asian countries sorbates are commonly used in combination with other preservatives to preserve fish sausages and similarly processed fish and meat products, as well as fermented plant foods (Chichester and Tanner, 1972; Lück, 1976, 1980; Robach and Sofos, 1982; Sofos, 1989). Several studies have reported improved preservation of various types of fish with sorbate (Geminder, 1959; Debevere and Voets, 1972; Robach and Hickey, 1978; Bremner et al., 1978; Chung and Lee, 1981; Lynch and Potter, 1982; Fey and Regenstein, 1982; Shaw et al., 1983; Bremner and Statham, 1983; Statham and Bremner, 1983; Regenstein, 1983; Sharp et al., 1986). One study, however, indicated that toxin production by *C. botulinum* in shucked scallops developed slightly more rapidly at 27°C in vacuum packages when sorbate (0.1%) was used (Fletcher et al., 1988). A sorbate (0.1%)–benzoate (0.1%) solution preserved brined shrimp for 59 days, which is longer than the preservation (31 days) achieved by the most effective bacteriocin (nisin) tested (Einarsson and Lauzon, 1995). Application of potassium sorbate with bifidobacteria extended the shelf life of whole and peeled shrimp by 3 days (Al-Dagal and Bazaraa, 1999). Sorbic acid at a concentration of 0.5% did not adversely affect the germination energies and germination capacities of sorghum grain and did not significantly affect malting loss, diastatic powers, and cold and hot water extracts of the malt samples. This optimal concentration of sorbic acid coincided with the lethal dosage for most of the associated fungal microorganisms during malting, inhibiting their growth; thus, it may be desirable to apply sorbic acid during malting (Ogundiwin et al., 1991).

The literature contains reports on several other food products that may be preserved with sorbates (Sofos, 1989). They include high-moisture marshmallow candy, sour cream, tangerine sherbet base, orange peels, strawberry puree, pineapples, tomato juice, Mexican-type hot sauces, prepeeled carrots, potato chips, cut squash, shredded cabbage, cucumbers, garlic oil, and pancake batter (Sofos, 1989).

OTHER APPLICATIONS

Other items that may be preserved by sorbic acid and its salts include pharmaceuticals, cosmetic products, and animal feeds (Sofos, 1989). In addition, the esters of sorbic acid (e.g., ethyl and propyl) may find applications in cosmetics and pharmaceuticals, especially those of the emulsion type, in which the emulsifiers may be inactivated by nonionic compounds (Chichester and Tanner, 1972; Lück, 1976). Sorbates may be particularly important in intermediate-moisture (25% moisture content) pet foods, in which levels of up to 0.30% can be effective mold inhibitors. Sorbic acid levels in the range of 0.03% to 0.10%, depending on moisture content, are also effective in preventing mold growth on animal feeds during storage. This should assure mycotoxin-free animal feeds and thus eliminate any hazards associated with mycotoxin-contaminated feed. Mayer and Hillebrandt (1997) established that chemical preservation with sorbic acid was a possible way of preventing potato pulp from spoiling for subsequent practical application (feed). Samples treated with sorbic acid lost biological activity within a few days of aerobic storage (confirmed by aerobic

degradation of starch and triglycerides). Although the presence of enzyme activities was shown, there was no detection of pectinolytic activities (Mayer and Hillebrandt, 1997). Blanching (80°C) and the application of 0.1% sorbic acid to citrus pulp were effective in inhibiting yeasts, molds, enterobacteria, and clostridia common in the spoilage of silage and, therefore, reduced ensiling losses and increased the feed value characteristics of citrus pulp silages (Filya et al., 2001). The preservatives sorbic acid and methyl paraben, when used individually or in combination as preservatives of an artificial diet for leafcutting bees, did not influence the susceptibility of the bees to the fungus *Ascosphaera aggregate* (Goettel and Duke, 1996). Other reported uses of sorbates include preservation of tobacco products, adhesives, and glues (Lück, 1976; Sofos, 1989).

MECHANISMS OF ACTION

Sorbate concentrations used in foods (<0.3%) usually inhibit microorganisms, whereas higher amounts may result in death. Consequently, when the sorbate hurdle is reduced or removed, the surviving microorganisms may resume growth and spoil the food. Sorbate inhibits cell growth and multiplication as well as germination and outgrowth of spore-forming bacteria, but the exact mechanisms of antimicrobial activity are not well defined (Freese et al., 1973; Freese and Levin, 1978; Sofos et al., 1986; Sofos, 1989).

Several studies have indicated that sorbate inhibits bacterial spore germination (Sofos et al., 1979d, 1986; Smoot and Pierson, 1981; Seward et al., 1982; Blocher and Busta, 1985). Inhibition has involved various species of bacteria in laboratory culture media and in foods and has been influenced by species, strains, pH, and sorbate concentration (Sofos, 1989). Published data have suggested that sorbate acts as a competitive and reversible inhibitor of amino acid-induced germination (Smoot and Pierson, 1981). Another study reported that sorbate inhibited spores triggered to germinate or after germinant binding (Blocher and Busta, 1985). It was also postulated that sorbate probably inhibits a postgerminant binding step in the process of germination, and it was thus concluded that it inhibits spore commitment to germination, and not triggering of germination. Inhibition of the not well-defined connecting reactions of spore germination may be taking place through the interaction of sorbate with spore membranes and through increases in their fluidity (Blocher and Busta, 1985; Sofos et al., 1986; Sofos, 1989).

Several mechanisms of inhibition of metabolic function by sorbate have been proposed, and it may be possible that several of them may be functional under various conditions, including types and species of microorganisms, type of substrate, environmental conditions, and the type of food processing. Under certain conditions sorbates have changed the morphology and appearance of microbial cells (Statham and McMeekin, 1988; Ronning and Frank, 1989; Sofos, 1989). Such changes have been observed in yeast cells as dense phosphoprotein granules, irregular nuclei, increased numbers and variable sizes of mitochondria, and vacuoles. Cells of *C. botulinum* were long, with bulbous formation and defective division (Seward et al., 1982; Wagner and Busta, 1985a,b). Sorbate-treated cells of *C. sporogenes* were usually filamentous and nonseptate but with distorted shapes characterized by numerous bends and bulges. Septation, when present, resulted in minicells, and the inner cell wall appeared to be thickened; the outer cell wall was absent in many areas (Ronning and Frank, 1989). Treatment of *Alteromonas putrefaciens* with sorbate at pH 7.0 increased cell hydrophobicity and cell wall lysis on exposure to lysozyme, which could be overcome to some extent by addition of magnesium ions. There was also evidence of other membrane damage in sorbate-treated cells (Statham and McMeekin, 1988). Although the significance of such alterations is unknown, they may be the result of the incorporation of sorbate into specific cell structures and alteration of biosynthetic processes in the cell (Sofos et al., 1986).

Other proposed mechanisms of inhibition of microbial growth by sorbate include alterations in the morphology, integrity, and function of cell membranes and inhibition of transport functions and metabolic activity (Sofos, 1989). Death of microorganisms exposed to high concentrations of preservatives, such as sorbate, has been attributed to generation of holes in the cell membrane

(Freese and Levin, 1978). Sorbate has decreased the assimilation of carbon from several substrates, including glucose, acetate, succinate, pyruvate, lactate, oxaloacetate, α -ketoglutarate, ethanol, and acetaldehyde (York and Vaughn, 1964; Harada et al., 1968; Sofos and Busta, 1981). Inhibition of cell metabolism by sorbate in these studies may have been the result of inhibition of enzymes, nutrient uptake, or various transport systems.

Sorbate inhibits the activity of several enzyme systems, which may lead to disruption of vital processes involved in transport functions, cell metabolism, growth, and replication. Enzymes inhibited by sorbate include alcohol dehydrogenase, fumarase, enolase, aspartase, catalase, malate dehydrogenase, α -ketoglutarate dehydrogenase, succinic dehydrogenase, and ficin (Melnick et al., 1954b; Whitaker, 1959; Azukas et al., 1961; Martoadiprawito and Whitaker, 1963; York and Vaughn, 1964; Troller, 1965). Some reports, however, have indicated no inhibition of enzymatic activity by sorbate (Sofos, 1989).

Sorbate is known to inhibit the *in vitro* activity of many enzymes, especially sulfhydryl-containing enzymes (Kouassi and Shelef, 1995a,b). Inhibition of sulfhydryl enzymes by sorbate has been attributed to binding the compound with sulfhydryl groups and decreasing the number of such active sites on the enzyme. Inhibition of yeast alcohol dehydrogenase has been attributed to formation of a covalent bond with the sulfhydryl or ZnOH group of the enzyme and the α - and/or β -carbon of sorbate (Martoadiprawito and Whitaker, 1963). It was also proposed (Whitaker, 1959) that sorbate inhibits the sulfhydryl enzymes through the formation of a thiohexenoic acid derivative ($\text{CH}_3\text{-CH=CH=RSCH-CH}_2\text{CO}_2\text{H}$). Inhibition of catalase by sorbate was attributed to the formation of sorbyl peroxides through the autoxidation of sorbic acid. These peroxides then would inactivate catalase (Troller, 1965). Another postulation has indicated that sorbate may act competitively with acetate at the site of acetyl coenzyme A (CoA) formation (Wakil and Hubscher, 1960; Harada et al., 1968; Sofos, 1992). Binding and inhibition of CoA should result in inhibition of oxygen uptake and microbial growth (Sofos, 1992).

Lipophilic acid food preservatives, such as sorbate, may interfere with substrate and electron transport mechanisms. Inhibition of substrate transport into the cell by uncoupling it from the electron transport system results in cell starvation (Deak and Novak, 1970; Sheu and Freese, 1972, 1973; Sheu et al., 1972, 1975; Freese and Levin, 1978). Sorbic acid has inhibited the uptake of glucose (Yousef and Marth, 1983) and amino acids (Hunter and Segel, 1973; Tuncan and Martin, 1985), as well as the electron transport system (Anderson and Costilow, 1963; Freese et al., 1973).

Inhibition of nutrient uptake may be the result of neutralization of the proton-motive force (PMF) needed for substrate uptake, inhibition of the electron transport system, inhibition of synthesis or depletion of ATP, inhibition of transport enzymes, and inhibition of metabolic energy utilization by the amino acid transport systems (Sofos, 1989). This effect may be occurring through incorporation of sorbic acid, or unsaturated fatty acid, into the cell membrane, where it may cause steric disorganization of active membrane transport proteins (Sofos, 1989, 1992). The highly reproducible and characteristic thermograms of *S. aureus* metabolism were significantly affected by sorbic acid in a concentration-dependent manner (Sayeed and Sankaran, 1991). Both the peak heat and total heat dissipation profiles were affected by 50% of the common concentration (0.2%) for use in foods. Additionally, the suggestion that inhibition of the membrane function is the primary site of action for sorbic acid is strengthened by an observation of damage to the outer cell membranes by sorbate ions at pH 7.0 in *Alteromonas putrefaciens* (Sayeed and Sankaran, 1991). Thus, microcalorimetric measurement of the effects of sorbic acid on *S. aureus* cells has clearly shown that the acid acts in its dissociated anionic form primarily on the substrate transport systems creating a starvation condition in the cells. Secondary events such as partial inhibition of the electron transport and inhibition of the activity or synthesis of catalase enzyme may have resulted in cell death (Sayeed and Sankaran, 1991). Sorbic acid could conceivably inhibit microorganisms as a weak-acid preservative, a membrane-active compound, or a specific inhibitor of metabolism (Azukas et al., 1961). There is evidence to suggest that protein denaturation or enhanced protein turnover is a further consequence of weak acid stress resulting from the expression of many proteins,

which act as molecular chaperones (de Nobel et al., 2001). Conversely, it has been shown that sorbic acid acts at high pH where weak-acid preservatives are not expected to be active (Stratford and Anslow, 1996). Sorbic acid in its MIC releases far fewer protons than other weak-acid preservatives. Similar degrees of inhibition by sorbic alcohol and sorbic aldehyde suggest that sorbic acid does not act as a weak-acid preservative. Specific inhibition of metabolism is also unlikely because inhibitory activity is shared by several small carbon compounds. Correlation of sorbic acid resistance with ethanol tolerance and the partition coefficient strongly suggest an inhibitory role for sorbic acid as a membrane-active compound (Stratford and Anslow, 1998).

Cells of *Z. rouxii* cultured in media supplemented with sorbate contained higher percentages of C18:1 fatty acids than cells cultured in media without sorbate (Golden et al., 1994). Because inhibition of microbial growth by sorbate has been associated with decreased levels of ATP (Harada et al., 1968; Hunter and Segal, 1973), a proposed mechanism of ATP depletion includes hydrolysis of ATP by the primary sodium/hydrogen pump in an attempt to maintain ion balance in the cell (Przybylski and Bullerman, 1980). As the hydrogen influx exceeds the pumped efflux, a shift in charge may potentially take place and lead to a decrease in the net negative intercellular charge. This could then discharge the pH gradient required for ATP formation according to the chemostatic theory of oxidative phosphorylation (Sofos, 2000). In another study using a novel method to measure pH in growing cells, little correlation was found between reduced growth on exposure to sorbic acid and reduction of intracellular pH (pH_i) (Bracey et al., 1998). In fact, growth inhibition correlated with an increase in the intracellular adenosine diphosphate (ADP)/ATP ratio as a result of increased ATP consumption by the cells. This was partly attributed to the activation of protective mechanisms, such as increased proton pumping by the membrane H^+ -ATPase, which ensured that pH_i did not decline when cells were exposed to sorbic acid (Henriques et al., 1997). Therefore, the available evidence suggested that the inhibitory action of sorbic acid was the result of the induction of an energetically expensive protective mechanism that compensated for any disruption of pH_i homeostasis but resulted in less available energy for normal growth. Thus, it is believed that the inhibitory action of sorbic acid is the result of excessive consumption of cellular energy that occurs as a consequence of the cell eliciting a stress response that attempts to maintain pH_i homeostasis such that the available energy for growth and cell division is drastically reduced (Bracey et al., 1998).

Evidence also suggests that this energy-demanding stress could also be the result of the induction of ATP-driven plasma membrane pumps for active extrusion of weak acids from the cell (Henriques et al., 1997). Indeed, as indicated, a pump recently identified as the Pdr12 protein is induced by sorbic acid in *S. cerevisiae* (Piper et al., 1998). Previous studies with bacteria, however, found no decreases in ATP in the presence of sorbic acid (Sofos, 1989, 1992, 2000). Inhibition of microbial growth by sorbate may be based on neutralization of the PMF that exists across the cell membranes by lipophilic, weak-acid preservatives such as sorbic acid and lead to starvation of cells from compounds that are transported actively by the PMF (Eklund, 1980, 1983, 1985; Ronning and Frank, 1987, 1988; Salmond et al., 1984; Sofos, 1989). In lower pH environments, the difference across the cell membrane is large, and the amount of acid entering and dissociating in the cytoplasm is higher. This accumulation of hydrogen ions inside the cell acts as an inhibitor by interfering with metabolic processes and causing a dissipation of the transmembrane proton gradient, which is one of the components of the PMF (Sofos, 2000). According to this theory, undissociated sorbic acid acts as a protonophore, which decreases the intracellular pH and dissipates the PMF of the membrane that energizes transport of compounds such as amino and keto acids. Inhibition of uptake of such components is believed to induce a stringent-type regulatory response in the cells, resulting in inhibition of growth but in maintenance of cell viability (Sofos, 2000). A stringent response involves readjustments occurring in bacteria when amino acids become limiting or their specific ratios are disturbed (Sofos, 2000).

Preincubation of *S. cerevisiae* yeast cells in the presence of benzoate or sorbate at an extracellular pH value of 6.8 elicited a set of metabolic effects on sugar metabolism, which became

apparent after the subsequent addition of glucose. These effects can be summarized as follows: (1) reduced glucose consumption; (2) inhibition of glucose- and fructose-phosphorylating activities; (3) suppression of glucose-triggered peak of hexose monophosphates; (4) substantial reduction of glucose-triggered peak of fructose 2,6-bisphosphate; and (5) block of catabolite inactivation of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxy-kinase, but not of cytoplasmic malate dehydrogenase (Burlini et al., 1993). On the whole, this pattern resulted in prevention of glucose-induced switch of metabolism from a gluconeogenic to a glycolytic state. Thus, unlike former assumptions, intracellular acidification is not likely to mediate the bulk of metabolic effects of benzoate and sorbate because under the described working conditions intracellular pH was kept close to neutrality (Burlini et al., 1993). Overall, it is believed that neutralization of the pH difference across the cell membrane is not the only mechanism of microbial inhibition by sorbate (Stratford and Anslow, 1998; Bracey et al., 1998; Sofos, 2000). Reasons for this conclusion include the following:

1. Although dissociation of the acid inside the cell may eliminate the pH difference across the membrane, its effect on the other component of the PMF, which is the difference in electrical potential, is smaller. However, it is believed to be adequate to energize the uptake of substances needed for cell maintenance and growth.
2. Sorbic acid has been shown to inhibit microbial growth, although less efficiently, even at pH values near neutrality; in such situations, uptake of the compound based on pH difference across the cell membrane would not be adequate to explain the observed antimicrobial activity.
3. Neutralization of the PMF is probably not involved in growth inhibition in the presence of carbohydrates because they do not depend on this force for their transport (Sofos, 2000).

Thus, the mechanisms involved in inhibition of microbial metabolism and proliferation by sorbic and other similar lipophilic acid preservatives appear to be different, depending on the type of microorganism, substrates, and environmental conditions (Sofos, 1989, 1992).

TOXICOLOGY AND SAFETY

In the United States, sorbate is considered a GRAS compound, and because it is a metabolizable fatty acid, the World Health Organization has set the acceptable daily intake for sorbate at 25 mg/kg body weight per day. The relative nontoxicity of sorbates was established early in their testing as food preservatives. The compounds were fed to various animal species for determination of acute toxicity, as well as influence on metabolism, carcinogenicity, and teratogenicity after short- or long-term exposure. Overall, these studies demonstrated the relative harmlessness of sorbates and their relative superiority in safety compared to other chemical additives.

Acute toxicity studies with rats have determined LD₅₀ (mean lethal dose) values for sorbates in the range of 4.2 to 10.5 g/kg body weight (Smyth and Carpenter, 1948; Deuel et al., 1954a). For comparison, the LD₅₀ for common salt (NaCl) is 5 g/kg body weight. Thus, based on acute toxicity, sorbate is considered one of the least harmful preservatives in use.

Rat feeding studies have indicated that a dosage of 10% sorbic acid in the diet could be tolerated for 40 days (Lück, 1980). On extending the feeding period to 120 days, however, the growth rate and liver weight of the animals increased (Demaree et al., 1955). Additional studies with rats and dogs have shown no damage with feeding 5% sorbic acid for 90 days, but 8% sorbic acid resulted in an increase in liver weight (Deuel et al., 1954a; Demaree et al., 1955; Lück, 1976). The increase in liver weight has not been associated with histopathologic changes and has been interpreted as functional hypertrophy as a result of the caloric utilization of sorbic acid (Deuel et al., 1954a; Food and Drug Research Laboratories, 1973; Lück, 1980; Sofos, 1989).

Chronic toxicity studies have involved feeding rats and mice through the life span of one or two generations with sorbic acid concentrations as high as 90 mg/kg body weight or diets containing 10% sorbic acid. No abnormalities have been observed, and in some instances the growth rate of sorbate-fed animals increased significantly, apparently because of an increased caloric intake resulting from the metabolizable sorbic acid. No carcinogenic or mutagenic effects have been observed with sorbate alone (Dickens et al., 1968; Shtenberg and Ignat'ev, 1970; Litton Biometrics, 1974, 1977; Food and Drug Research Laboratories, 1975; Gaunt et al., 1975; Hendy et al., 1976; Lück, 1980; Sofos and Busta, 1981, 1993; Sofos, 1994).

An allergic-type response has been reported for sorbic acid in one study in which bacon samples with sorbate–nitrite combinations were tested against *C. botulinum*. Some taste panelists reported certain “allergic-type” symptoms after tasting uninoculated experimental bacon (U.S. Department of Agriculture, 1979b; Berry and Blumer, 1981). Although it was implied that sorbate might have been involved in producing those symptoms, no direct relation could be proved between symptoms and specific ingredients used in formulating the bacon (U.S. Department of Agriculture, 1979b). In addition, no such symptoms were observed by other individuals who tasted sorbate–nitrate experimental bacon from other studies, and Robach and Adam (1980) were able to induce such symptoms from panelists consuming commercial bacon manufactured without sorbate. This, of course, does not rule out the possibility that high concentrations of sorbate may act as irritants to certain susceptible individuals (Sofos, 1989).

As preservatives for cosmetics and pharmaceutical products, sorbates have been extensively examined for skin tolerance (Lück, 1976; Patrizi et al., 1999). The literature is contradictory, but it indicates that most people are not affected by sorbic acid applied to the skin. Some sensitive individuals, however, show skin irritations when exposed to sorbic acid (National Academy of Sciences, 1982; Dejobert et al., 2001). Average sorbic acid concentrations for skin irritations are in the range of 1%, and some very sensitive individuals may show irritations even at lower levels (Lück, 1976). Considering the average use levels of 0.10% to 0.30% in food processing, the potential for such irritations in commercial products is minor.

There has been some concern about possible sorbate–nitrite reactions when added to the same substrate (Sofos, 1981; National Academy of Sciences, 1982). Because there is evidence of reactions between nitrite and fatty acids (Benedict, 1980) and because sorbic acid is an unsaturated fatty acid, it may react with nitrite in products such as cured meats. The products of such reactions have the potential of being mutagenic (Sofos, 1989). In general, however, various studies have indicated the following: (1) nitrite reacts with sorbic acid to form mutagens optimally at pH 3.5, not at 6.0, which is the average pH of most meat products; (2) formation of the c-nitroso mutagens requires the presence of excess nitrite; and (3) mutagen formation is inhibited by such ingredients as ascorbic acid, cysteine, and vegetable juices and is inactivated by heat (Kada, 1974; Kito et al., 1978; Osawa et al., 1979, 1980, 1982, 1986; Robach et al., 1980a; Namiki, 1979, 1980, 1981, 1983; Khoudokor-moff, 1981; Osawa and Namiki, 1982). Thus, the requirements for the reactions to proceed and the instability of the products make it unlikely for mutagens to be formed at detectable or harmful concentrations in foods treated with nitrite and sorbate (Sofos, 1981, 1989). Walker (1990) concluded that sorbate use, in general, does not appear to create toxicologic problems.

It has been regarded that amino-compounds, including lysine and glutamate, may undergo nonenzymatic browning reactions when stored with sorbic acid at a favorable range of a_w and temperature (Quattrucci and Masci, 1992). This reaction was demonstrated in model systems but, to some extent, it may apply also to more complex systems such as foods, leading to decreased amino acid availability (Quattrucci and Masci, 1992). Sorbates were found to react easily with proteins, forming high-molecular-weight products that do not seem to result in a nutritional loss, probably because the acid environment of the stomach leads to the breakdown of the sorbic-protein adducts (Quattrucci and Masci, 1992).

Sorbic acid has a conjugated system of double bonds, which makes it susceptible to nucleophilic attack, sometimes giving mutagenic products (Ferrand et al., 2000a,b). Under conditions typical of

food processing (50°C to 80°C) cyclic derivatives resulting from a double addition reaction between sorbic acid and various amines were analyzed. The formation of new products by addition reactions using ethyl sorbate and various amines led, at 20°C, to linear monoadducts and, at 50°C, to cyclic derivatives resulting from double addition (Ferrand et al., 2000a,b). Mutagenesis studies, involving the Ames test and genotoxicity studies with HeLa cells and on plasmid DNA, in cyclic interaction products, showed that none of the products studied presented mutagenic or genotoxic activities (Ferrand et al., 2000a,b). It should be noted, however, that certain studies have reported inhibition by sorbate of carcinogenic nitrosamine formation in model systems (Tanaka et al., 1978; Chung, 1981; Lathia and Schellhoeh, 1981; Massey et al., 1982; Rao et al., 1982; Yamamoto et al., 1988). Sorbic acid, however, had no effect on nitrosamine formation from aminopyrine and sodium nitrite in animal stomachs (Kawanishi et al., 1981a,b; Sofos, 1989). Potential injury in cell hepatocytes induced by potassium sorbate was prevented by antioxidants (Sugihara et al., 1997).

No synergism in acute toxicity has been detected for combinations of sorbic acid with various other additives, including parabens, benzoate, and propionate (Sofos, 1989). Sorbate, however, did not protect against the toxic effects of other substances (Daoud and Griffin, 1980). The derivatives of sorbic acid are also relatively nontoxic. Overall, sorbates appear to be one of the safest food preservatives available.

DETECTION AND ANALYSIS

Analytic methods used or tested for qualitative and quantitative detection of sorbates in foods include acidimetry, bromometry, colorimetry, enzymatic, spectrophotometry, polarography, and chromatography. The most widely used methods, however, have been colorimetric (Schmidt, 1960, 1962) and spectrophotometric (Melnick and Luckman, 1954a), although chromatographic methods have gained acceptance in recent years.

Detection methods require quantitative extraction and separation of sorbic acid from the food material without food ingredient interference (DeLuca et al., 1995; Mandrou et al., 1999; Montano et al., 1995; Sofos, 2000). Extraction methods include acid-steam distillation, selective gas diffusion, and solvent extension using ethyl or petroleum ether, dichloromethane, and isooctane. In some foods, filtration, dialysis, or direct analysis has been used (Sofos, 1989).

Extraction by steam distillation has been used extensively, but it is time consuming and the compounds present in the food or generated by decomposition of lipid materials may interfere with colorimetric or spectrophotometric detection of sorbic acid (Harrington et al., 1962; Sofos, 1989). Several modifications have been proposed as useful in avoiding interference and in improving the accuracy of the steam distillation procedure (Luckmann and Melnick, 1955; Sofos, 1989), whereas combinations of various treatments have also been used to improve extraction and reduce interference (Sofos, 1989). Such combinations have involved extraction under acid conditions from the steam distillate and reextraction with sodium hydroxide; dialysis and solvent extraction; successive extractions with ether, sodium hydroxide, and methylene chloride; and double distillation and ether extraction (Tjan and Konter, 1972; Noda et al., 1973; Larsson, 1983; Puttermans et al., 1983; Sofos, 1989).

The colorimetric detection of sorbic acid at an absorbance of 532 nm is an official method for quantitative determination in foods and beverages (AOAC Int. method 975.31), cheese (AOAC Int. methods 971.15 and 975.22), and wine (AOAC Int. method 975.10) by the color reaction with α -thiobarbituric acid (Roy et al., 1976; AOAC Int., 2000). The method is simple and usually very specific (Lück, 1980). The spectrophotometric (ultraviolet/UV, absorption) procedure has also been used in many foods, including fruit products, bakery items, wine, cheese, and sausage products (Maxstadt and Karasz, 1972; Wilamowski, 1974; Stafford, 1976; Holley and Millard, 1980). After development by Melnick and Luckman (1954a), the method was modified by Alderton and Lewis (1958); it involves measurement of absorbance at 260 nm (250 to 290 nm). Special extraction and purification steps have been proposed in the literature to reduce interference problems (Sofos,

1989). The method is an official first action procedure for ground beef (AOAC Int. method 980.17) and final action procedure for wines (AOAC Int. method 974.08) and dairy products (AOAC Int. method 974.10) (AOAC Int., 2000).

The literature contains numerous reports of chromatographic methods used or evaluated for determination of sorbate in food products (Sofos, 1989). They include gas chromatography, high-performance liquid chromatography (HPLC), thin-layer chromatography, paper chromatography, and micellar electrokinetic capillary chromatography (MECC) and ion chromatography. A gas chromatographic method is a first action procedure (AOAC Int. method 983.16) for sorbic and benzoic acids in foods (AOAC Int., 2000). Solid extraction has been used as a cleanup procedure for the determination of sorbic acid by liquid chromatography in fruit products (Mandrou et al., 1999). Others, however, have reported that cleanup procedures did not improve determination by liquid chromatography (Benassi and Cecchi, 1998). Microdialysis was used to extract sorbic acid and benzoic acid from food to be separated and detected by HPLC (Mannino and Cosio, 1996). In addition, HPLC was found to be adequate in obtaining accurate stability data for sorbic acid in creams (de Villiers and Bergh, 2000). A rapid method for the identification and quantitation of sorbic and benzoic acids in beverages and foods by MECC has been reported (Pant and Trenerry, 1995). Ion chromatography has been shown to be in good agreement with HPLC in determinations of sorbic acid in food and pharmaceutical preparations (Chen and Wang, 2001). In addition, ion chromatography was effective in simultaneously determining artificial sweeteners, preservatives, caffeine, theobromine, and theophylline and as such may be a beneficial alternative to conventional HPLC.

A polarographic method using differential-pulse voltammetry with the use of a hanging mercury drop electrode (HMDE) was developed for the determination of sorbic acid in fruit juices and drinks. The procedure is simple and specific and is not subject to interference from many substances commonly found in soft drinks such as ethanol and benzoic acid. Moreover, it can be used for the analysis of intensely colored juice samples. Thus, the scope of application of the proposed method for the determination of sorbic acid has been extended to cover juice and drink samples that cannot be analyzed by the AOAC procedure (Fung and Luk, 1990). The use of reversed-phase high-performance liquid chromatography (RP-HPLC) was found to be a simple, rapid method with high reproducibility for determining sorbic acid and other additives in fruit juices (Zou et al., 2001).

An enzymatic method used to determine sorbic acid based on the spectrophotometric measurement of sorbyl CoA at 300 nm has been developed (Hofer and Jenewein, 2000). The method is based on sorbic acid being converted to sorbyl CoA with acyl CoA synthetase in the presence of coenzyme A and adenosine-5'-triphosphate. The reaction is quantified by irreversibly hydrolyzing pyrophosphate to phosphate in the presence of inorganic pyrophosphatase, where the absorbance is measured at 300 nm and is specific for sorbyl CoA, which is proportional to sorbic acid levels in the sample. The method was found to be fast, precise, and reliable and is thus well suited for routine determinations, especially for high sample throughputs (Hofer and Jenewein, 2000).

Capillary electrophoresis was also applied for detection in citrus juices, wine, and other substrates (Cancalon, 1999; Mercier et al., 1998; Castineira et al., 2000; Dobiasova et al., 2002). Inhibition of microorganisms has also been evaluated as a procedure for the qualitative detection of sorbate (Ellerman, 1977). It is obvious, however, that in addition to sorbate, such inhibition may be caused by a number of other inhibitors.

REGULATORY STATUS

Several forms of sorbate are allowed for use in a variety of foods throughout the world (Sofos, 1989). This major use may expand in the future, considering their extensive testing, low toxicity, and advantages over other preservatives. In the United States, sorbic acid and potassium sorbate are GRAS, and this status has been reaffirmed (U.S. Department of Agriculture, 1978) by a select committee of the U.S. Food and Drug Administration. In addition, sorbates in the United States

are sanctioned in more than 80 food products designated by standards of identity. According to the Code of Federal Regulations, when a food preservative is used in a food product, its common name (e.g., potassium sorbate or sorbic acid) should be listed on the product label and its function should be indicated by an explanatory description (e.g., “to maintain freshness,” “to extend shelf life,” or “as a preservative”). The use of sorbates may be requested for any food product that allows preservatives.

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4 Organic Acids

Stephanie Doores

CONTENTS

Effective Use	93
The Acids	94
Acetic Acid	94
Antimicrobial Properties	94
Toxicology	99
Application and Regulatory Status	101
Acetates	101
Antimicrobial Properties	101
Toxicology	102
Application and Regulatory Status	103
Sodium Diacetate	103
Antimicrobial Properties	103
Toxicology	104
Application and Regulatory Status	104
Dehydroacetic Acid.....	104
Antimicrobial Properties	104
Toxicology	105
Application and Regulatory Status	105
Adipic Acid	105
Antimicrobial Properties	105
Toxicology	105
Application and Regulatory Status	106
Caprylic Acid	106
Antimicrobial Properties	106
Toxicology	106
Application and Regulatory Status	106
Citric Acid.....	107
Antimicrobial Properties	107
Toxicology	110
Application and Regulatory Status	110
Fumaric Acid.....	111
Antimicrobial Properties	111
Toxicology	111
Application and Regulatory Status	112
Lactic Acid	112
Antimicrobial Properties	112
Toxicology	116
Application and Regulatory Status	116

Lactates	116
Antimicrobial Properties	120
Application and Regulatory Status	120
Malic Acid.....	120
Antimicrobial Properties	120
Toxicology	120
Application and Regulatory Status	120
Propionic Acid	120
Antimicrobial Properties	120
Toxicology	122
Application and Regulatory Status	123
Succinic Acid	123
Antimicrobial Properties	123
Toxicology	123
Application and Regulatory Status	123
Tartaric Acid.....	124
Antimicrobial Properties	124
Toxicology	124
Application and Regulatory Status	124
Mode of Action	124
Acid Adaptation and Resistance.....	126
Assay	127
References	127

Many parameters govern the survival and growth of microorganisms in food. The acidity or pH of a food can affect the type and number of microorganisms present in a product. All microorganisms have minimum, maximum, and optimum pH levels for growth, and altering the hydrogen ion concentration influences the growth or inhibition of an organism. In general, bacteria are more fastidious and prefer to grow at a pH near neutrality (pH 6.5 to 7.5), but they will tolerate a pH range of 4 to 9. Yeasts are more tolerant of lower pH values than bacteria. Molds have the widest range of acceptable pH; foods with a pH below 3.5 can support the growth of both yeasts and molds. Tolerance of organisms to widely differing pH levels varies naturally, and the pH selects the species or group of microorganisms that will predominate in unaltered food products. For example, bacteria primarily spoil proteinaceous foods such as dairy, meat, poultry, and seafoods with a pH range of 5.5 to 6.5. Yeasts and molds more commonly proliferate on fruits and vegetables with inherently lower pH values and little buffering capacity.

One effective means of limiting growth is to increase the acidity of a food, thereby creating an unfavorable environment. Adding an acidulant to the food or enhancing natural fermentation to develop acidity changes the pH of the food. These actions tend to be microbiostatic rather than microbiocidal. Success in limiting the numbers of microorganisms will depend on the species of microorganism, the type and concentration of the acidulant, time of exposure, the buffering capacity of the food, and any preexisting conditions in the food that could enhance inhibition.

Microorganisms display varied tolerances to acids. For example, the lactic acid bacteria are not only tolerant of weak lipophilic acids but also produce them as a by-product of their metabolism. Some acids, such as acetic acid, are critical to the metabolism of the lactobacilli but inhibitory to bacilli. In mixed flora, the proper use of an acid in a culture medium can select for a particular group.

The incorporation of acids into a food can shorten sterilization times for heat treatment owing to the lowered heat resistance of microorganisms in foods with increased acidity. The continued presence of acid can effectively inhibit germination and outgrowth of spores that survive the thermal process. Salt, sugar, and curing agents in conjunction with acids serve to further decrease processing

TABLE 4.1
Dissociation Constants of Organic Acids in Aqueous Solutions

Acids	pK ₁	pK ₂	pK ₃
Acetic acid	4.75		
Dehydroacetic acid	5.27		
Sodium diacetate	4.75		
Adipic acid	4.43	5.41	
Caprylic acid	4.89		
Citric acid	3.14	4.77	6.39
Fumaric acid	3.03	4.44	
Lactic acid	3.08		
Malic acid	3.40	5.11	
Propionic acid	4.87		
Succinic acid	4.16	5.61	
Tartaric acid	2.98	4.34	

Source: From Lide (1991).

times. Not only would this interaction ensure commercial sterility of the food, but also the decreased processing time would aid in preserving the palatability of the product (Corlett and Brown, 1980).

The multiple-barrier concept (“hurdle” concept) has gained popularity for use in a variety of foods in recent years. This concept is based on the premise that foods can be preserved using several inhibitors concurrently rather than relying on a single factor. Therefore, adjustments in pH levels can be coupled with changes in temperature, gaseous atmosphere, water activity, and other inhibitory compounds. The use of the multiple-barrier concept results in lowering the concentrations of acidulants or inhibitors.

Comprehensive texts describing applications, physical parameters, and chemical analyses can be found in the references by Ash and Ash (1995) and Doores (2002, 2003).

EFFECTIVE USE

Various approaches have been used to study the efficiency of antimicrobial effects on cells. The inhibitory effect of acids has been compared based on pH, concentration, chain length, type, and degree of branching to inhibit or kill a wide variety of microorganisms. Because some experiments express concentration in percent, normality, or molarity to achieve final pH, it is somewhat difficult to compare acids or make broad statements concerning the choice of the appropriate acid for a particular effect in a category of foods.

Effective use of an acidulant depends on the dissociation constant (pKa) or the pH at which 50% of the total acid is dissociated. The pKa of most organic acids lies between pH 3 and 5 (Table 4.1). Because the undissociated portion of the molecule is believed to be responsible for the antimicrobial effect, it would be advantageous to use the acids near these values. This primary concern limits the use of acidulants to foods with pH values of less than 5.0. Given that this value lies at the lower limits of growth for many bacteria, organic acids are usually more effective as antimycotic agents. Hoffman et al. (1939) observed that acids with fewer than 7 carbons were more effective at lower pH, and acids with 8 to 12 carbons were more effective at neutrality and above.

In addition to any antimicrobial effects an acid possesses, the choice of an acidulant may depend on secondary effects. Acids contribute to the taste and tartness of a product. They can create a synergistic relationship with antioxidants by chelating metal ions. They can control pectin gel formation, aid in the inversion of sucrose, prevent browning, and protect color. Some can be used

as chemical leavening agents. The salts of the acids are important in regulating the acidity of the foods (Gardner, 1966)

The toxicology and safety of the acids must be considered when selecting an acid for use. Most of the acids appear safe for use in food products. Several animal studies have indicated possible safety hazards; however, many of these studies used high levels of the concentrated acids at low pH values. At the levels and pH values in which these acids are normally used in foods, the same hazards would not be expected. The long-term consumption of these acids, which exist naturally in many foods, would further indicate that hazards are unlikely. In addition, all these acids can be metabolized by the body primarily through lipid oxidation and via the tricarboxylic acid cycle.

THE ACIDS

Information on the toxicology, antimicrobial properties, application, and regulatory status is presented here for each acid. The lipophilic acids include acetic acid and acetic acid derivatives, propionic acid, and caprylic acid. These acids, as the name implies, are relatively nonpolar compounds. They are usually added to foods for their antimicrobial properties, although they could also serve other functions in foods. The carboxylic acids are more polar than the lipophilic acids and are traditionally used in foods for secondary effects rather than for their ability to inhibit microbial growth.

Numerous studies included in this chapter compare several organic acids for their antimicrobial effects. The results of these studies are usually presented in the section describing the acid that gave the most antimicrobial effects. In some instances, the information appears separately for each acid.

ACETIC ACID

Antimicrobial Properties

Acetobacter and heterofermentative lactic acid bacteria (heterolactics) produce acetic acid as a by-product of their metabolism and, as such, are more tolerant to this acid than homofermentative lactic acid bacteria (homolactics), which do not. These organisms are found naturally associated with fermented products, such as pickles, sauerkraut, and vinegar.

Adams and Hall (1988) showed a weakly synergistic inhibitory effect between acetic and lactic acids with *Salmonella enteritidis* and *Escherichia coli*. Although these acid mixtures might be expected to occur naturally during fermentations by heterolactic bacteria, this apparent synergy has not been demonstrated in other foods. This association could explain the apparent stability that occurs in natural fermentations, such as sauerkraut. Sauerkraut manufacture is a fermentation process brought about by a natural succession of microbes that inhabit the surface of cabbage leaves. The succession typically begins with growth of coliforms and natural microflora followed by *Leuconostoc mesenteroides* (heterolactic), then by *Lactobacillus plantarum* (homolactic), a more acid-tolerant organism. McDonald et al. (1990) found that growth of *L. mesenteroides* ceased when the internal pH of the cells reached 5.4 to 5.7, whereas growth stopped in *L. plantarum* at pH 4.6 to 4.8. *L. plantarum* maintained its pH gradient in the presence of either 160 mM sodium acetate (SA) or sodium lactate down to an external pH of 3.0.

Microorganisms vary in their susceptibility to acetic acid. Concentrations of acid lower than those needed to inhibit *Saccharomyces cerevisiae* (pH 3.9) and *Aspergillus niger* (pH 4.1) (Levine and Fellers, 1939) inhibited *Bacillus cereus* (pH 4.9), *Salmonella Aertrycke* (pH 4.9), and *Staphylococcus aureus* (pH 5.0). *Bacillus* and *Clostridium* species and Gram-negative bacteria were more inhibited at pH 6.0 than lactic acid bacteria, Gram-positive bacteria, yeasts, and molds; however, as the pH decreased to 4.0, all of the groups were similarly affected (Woolford, 1975b). Owen (1946) suggested that acetic acid inhibited Gram-positive organisms.

S. aureus was most sensitive to acetic, followed by citric, lactic, malic, tartaric, and hydrochloric acids (Nunheimer and Fabian, 1940). Acetic acid exerted the most inhibition but had the lowest dissociation constant, whereas tartaric acid had one of the highest dissociation constants yet exerted the weakest inhibitory action. Similar results were noted when various acids were compared at the pH level required for a 90% and 99% reduction in numbers of *S. aureus* in 12 hours (Minor and Marth, 1970). Acetic acid was effective at pH 5.2 and 5.0, respectively, followed by lactic, pH 4.9 and 4.6; citric, pH 4.7 and 4.5; and HCl, pH 4.6 and 4.0. Both studies concluded that the antimicrobial effect was the result of the undissociated molecule. *S. aureus* preconditioned in acetate buffer (pH 4.4 to 4.6) at 20°C shifted to pH 7.0 buffer, and exposed to 40°C became injured, but no injury occurred in cells not exposed to acidic conditions before heating (Smith et al., 1984).

B. cereus was inhibited at pH 5.0 with 0.15 M acetic acid and with 0.33 M lactic acid (Wong and Chen, 1988). A 0.1-M concentration of acetate and lactate completely inhibited multiplication at pH 6.1 and 5.6, respectively, and caused 50% inhibition of spore germination at pH 4.2 and 4.3, respectively.

Buffered acidulant systems (brain heart infusion [BHI] broth, pH 4.8, 22°C) containing 1.0% acetic acid and 1.0% lactic, malic, tartaric, or citric acid were far more effective in reducing numbers of *S. aureus*, *Salmonella* Blockley, *Streptococcus faecalis*, and *E. coli* than unbuffered systems. This finding is important in the manufacture of medium acid foods containing more than one organic acid, such as mayonnaise (Debevere, 1988).

E. coli O157:H7 is considered more acid tolerant than strains of *E. coli*. When *E. coli* O157:H7 was inoculated into tryptic soy broth (TSB) containing acetic, citric, lactic, malic, and tartaric acids, growth occurred at pH 5.5 for all acidified media at 10°C except for acetic acid. At 25°C and 37°C, *E. coli* O157:H7 grew in all media at pH 5.0 with scant growth in TSB containing acetic acid. Malic acid allowed growth at pH 4.5, and tartaric acid permitted growth at pH 4.0 (Conner and Kotrola, 1995; Conner et al., 1997).

Traditionally acidic foods contain a single or multiple acids, added or developed through fermentation. In addition, other ingredients can interact with acid to influence the degree of inhibitory action. Reduced-calorie mayonnaise (RCM) manufactured with 0.1%, 0.3%, 0.5%, or 0.7% acetic acid in the aqueous phase and adjusted to pH 4.0 with hydrochloric acid was compared to cholesterol-free (egg yolk-free) reduced-calorie mayonnaise (CFM) manufactured with 0.3% and 0.7% acetic acid (Glass and Doyle, 1991). Both formulations were inoculated with approximately 10⁸ CFU/ml of an 8-strain mixture of *Salmonella* or a 6-strain cocktail of *Listeria monocytogenes* and held at 23.9°C. *Salmonella* were inactivated within 48 hours in both RCM and CFM formulated with 0.7% acetic acid with reduction to undetectable levels in 1 and 2 weeks with 0.5% and 0.3% acetic acid formulations, respectively. Destruction of *Salmonella* occurred more quickly in CFM than RCM, most likely because of the absence of egg yolks in CFM and the presence of egg white, which has recognized antimicrobial properties. *L. monocytogenes*, although more resistant than *Salmonella*, was reduced by 4 logs in both mayonnaises within 72 hours in formulations containing 0.7% acetic acid. *L. monocytogenes* was also reduced to undetectable levels in RCM within 10 days and in CFM within 14 days compared to 2 days for *Salmonella*. It is unlikely that these products would contain such high initial levels of either of these organisms, especially with the use of pasteurized egg products, therefore the standard use of 0.7% acetic acid, pH 4.0, in the aqueous phase of CFM and RCM would still produce a microbiologically sound product.

A concentration of 0.4% to 0.8% reduced growth of *Micrococcus* and *Bacillus* species, some strains of *S. aureus*, Gram-negative aerobic bacteria, and Enterobacteriaceae in Japanese fermented soy sauce (Hayashi et al., 1979). An increased toxic effect of acetic acid against *Saccharomyces rouxii* and *Torulopsis versatilis* was demonstrated in brine fermentation of soy sauce as the pH decreased from 5.5 to 3.5 (Noda et al., 1982).

The effect of temperature-acid interactions is noteworthy for the psychrotrophic pathogens, *L. monocytogenes* and *Yersinia enterocolitica*. Acetic acid caused greater inactivation of *L. monocytogenes* than lactic and citric acids and increased inhibition as the temperature of incubation

decreased from 35°C to 13°C; concentration $\geq 0.3\%$ inhibited *L. monocytogenes* irrespective of temperature. However, citric acid caused greater injury than either lactic or acetic acids and injured organisms survived longer at low temperatures (Ahamad and Marth, 1989, 1990).

Acetic acid displayed the greatest antimicrobial action against *L. monocytogenes* when compared to citric, malic, lactic, and hydrochloric acids at equal pH values and at all incubation times and temperatures (Sorrells et al., 1989). When comparisons were based on equal molar concentrations, however, citric acid was more bactericidal than other acids at 25°C and 35°C, but malic acid was more bacteriostatic than other acids at 10°C. The greatest antimicrobial effect occurred at 35°C with the greatest survival at 10°C. The minimum inhibitory pH level was pH 4.4 for malic, citric, and hydrochloric acids, pH 4.4 to 4.6 for lactic acid, and pH 4.8 to 5.0 for acetic acid. Sorrells and Enigl (1990) also demonstrated interactive effects between sodium chloride, acidulants, and temperature.

Similar growth inhibition was demonstrated for *Y. enterocolitica* at lower pH levels as the temperature increased. Acetic acid was more effective than lactic or citric acids in producing this effect (Adams et al., 1991; Brocklehurst and Lund, 1990; Karapinar and Gönül, 1992a; Little et al., 1992). Conversely, injury and death rates were not affected by refrigeration temperatures for *Salmonella* Bareilly, a mesophilic organism. A 90% reduction of the population required exposure to 0.01 N for 75 min, 0.05 N for 8.5 min, 0.10 N for 5 min, and 0.2 N for 4 min. Acid injury did not appear to involve damage to ribosomes or other nucleic acid material, but recovery required the presence of amino acids or peptones (Blankenship, 1981).

Raw produce is now being subjected to acid rinses in some production practices as a means of reducing high levels of spoilage organisms and foodborne pathogens. Because produce is typically refrigerated with only minimal processing, these foods can be the carriers of psychrotrophic pathogens, such as *Y. enterocolitica*. Parsley, inoculated with approximately 10^7 CFU/g *Y. enterocolitica*, was dipped in acetic acid solutions (1%, 2%, or 5%) or vinegar (30%, 40%, or 50%) for 15 or 30 minutes. *Y. enterocolitica* was not detectable after exposure to 2% and 5% acetic acid or 40% and 50% vinegar for 30 minutes (Karapinar and Gönül, 1992b).

Acetic acid applied as a volatile compound (1000 mg/L of air) for 7 hours at 60°C achieved >3 log reduction of six *Salmonella* strains inoculated onto alfalfa seeds compared to 1.9 log reduction for the nontreated seeds (Weissinger et al., 2001). Exposure at 50°C for 12 hours with lower levels (100 and 300 mg/L of air) reduced the population >1.7 log without affecting germination of seeds. Treatment at low temperature (4 days at 10°C) with 200 and 500 mg/L of air was effective in reducing *Salmonella* by 2.33 and 5.72 logs, respectively, but resulted in changes in sensory characteristics. Mung bean seeds inoculated with 3 to 5 logs of *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* were exposed to acetic acid at a concentration of 242 $\mu\text{L/L}$ of air for 12 hours at 45°C. This treatment reduced *S. Typhimurium* and *E. coli* O157:H7 to nondetectable levels by enrichment methods, but *L. monocytogenes* was isolated from 2 of 10, 25-g samples. In addition, vapor treatment also reduced levels of resident microflora on seed but did not affect seed germination rates (Delaquis et al., 1999). Immersion of alfalfa seeds in mixtures containing 5% lactic or citric acid for 10 minutes resulted in several log reductions, but acetic acid was less effective. In addition, seed germination was effected (Weissinger and Beuchat, 2000).

A variety of organic acid treatments has been used to control microbial loads associated with meat carcasses and products (Dickson and Anderson, 1992; Dorsa, 1997; Dorsa et al., 1997, 1998; Smulders and Greer, 1998). Numerous organic acid combinations, containing from 0.6% to 3.0% acetic acid at pH levels from 1.5 to 3.0 have been used to rinse pork, lamb, and beef carcasses or meat tissue. Treatments spanned varying amounts of time with concomitant decrease in microbial loads up to 3 logs. Depending on the treatments, there can be adverse sensory effects including discoloration of meat, off-odors, and off-flavors.

Pork carcasses inoculated with 10^6 CFU/ml *S. Enteritidis* were sprayed with acetic acid at pH 1.5 or 2.0 for 30 or 60 seconds (Biemuller et al., 1973). Total plate counts were reduced by 4 logs at pH 1.5, a twofold reduction at pH 2.0, 1 log unit at pH 2.5, and no reduction in microbial

numbers at pH 3.0. The treatment effectively reduced recovery of *S. Enteritidis* to 1 of 6 samples at pH 1.5 and 11 of 72 for pH 2.0. Although washing of pork carcasses with 1.5% acetic, citric, or lactic acid led to significant decreases in aerobic plate and coliform counts for acetic and citric acid washes after 14 days of storage at 2°C to 4°C, *L. monocytogenes* was detected in 69% of loins and 33% of chops, suggesting that acids had limited effectiveness (Fu et al., 1994). Increasing the concentration to 2% acetic acid or using 200-ppm hypochlorite solutions before vacuum packaging and storage for 28 days at 4°C significantly lowered aerobic, anaerobic, and lactic acid bacteria counts, however, some discoloration occurred (Cacciarelli et al., 1983). A 3% concentration of acetic acid was quite effective in reducing counts of Enterobacteriaceae in vacuum-packaged pork stored for 6 weeks at 2°C to 4°C (Mendonca et al., 1989b). An application of 2% acetic acid reduced the incidence of *Salmonella* on pork cheek meat in addition to significantly reducing aerobic plate and coliform counts (Frederick et al., 1994). An 18% acetic acid or 12% lactic acid spray significantly reduced bacterial counts on lamb carcasses. At this concentration, the pH level decreased to 2.1 and 1.5, respectively, and bleaching of the carcasses occurred (Ockerman et al., 1974).

As part of recently established guidelines by the U.S. Department of Agriculture Food Safety Inspection Service (FSIS), organic acids can be used in preevisceration rinse systems consisting of a water rinse followed by an organic acid rinse. Application of a 3% concentration of acetic acid as a spray on beef carcasses was not significantly better than water washes in reducing levels of *E. coli* O157:H7; however, a final application of acid washes could provide some residual effect during storage (Brackett et al., 1994; Cutter et al., 1997; Cutter and Siragusa, 1994; Dorsa et al., 1997; Hardin et al., 1995).

Quartey-Papafio et al. (1980), found that a 1% formic and 1% acetic acid combination exposed to beef for up to 20 minutes was effective in reducing a variety of bacterial species. Beef cubes treated with 1.2% acetic acid or 0.6% acetic and 0.046% formic acids for 10 seconds caused discoloration from both treatments, but beef treated with the mixture of acids did not differ in flavor from untreated beef (Bell et al., 1986). Hamby et al. (1987) used intermittent sprays of 1% acetic acid or lactic acids, which resulted in significant reductions in aerobic plate count (1.8 to 4.3 log/cm²). Meat was sprayed, vacuum packaged, and stored for 28 days at 2°C in a high-oxygen barrier film. *Lactobacillus* species predominated as storage progressed. Application of a 2% lactic or 2% acetic acid spray to beef strip loins and stored at 1°C resulted in significantly lower aerobic and lactic acid bacteria counts over 112 days of storage (Goddard et al., 1996).

Reductions in microbial counts depend on the water temperature, type of chemicals, and application rate and sequence in the process (Gorman et al., 1995). Anderson et al. (1977) reduced bacterial counts by 99.6% on meat using a 3% concentration of acetic acid before washing. They found that acetic acid was a superior sanitizer compared to hypochlorite and had a greater residual effect. Further work by Anderson and Marshall (1989) demonstrated that application of acetic acid at 70°C was most effective in sanitizing beef semitendinous muscle inoculated with *E. coli*, *S. Typhimurium*, or manure slurry. Temperature played a greater role in reducing numbers than did the acid concentration. Microbial loads on beef carcasses subjected to machine washing and sanitization with 1.5% acetic acid were reduced more when treatments were conducted at 52°C than at 14.4°C (Anderson et al., 1987). Incorporation of pulsed power electricity and a 2% acetic acid spray led to improved reduction of inoculated *E. coli* O157:H7 and *S. Typhimurium* on beefsteaks (Tinney et al., 1997).

Dickson (1992) contaminated lean and beef tissue surfaces with *S. Typhimurium* followed by treatment with 2% acetic acid. *S. Typhimurium* was reduced by 0.5 to 0.8 log CFU/cm²; however, this was not significantly different from the controls. *S. Typhimurium* reductions were greater when the bacteria were attached to fatty tissue, possibly because this tissue retained less moisture (20%) than lean tissue (75%) and the reduced water activity could have enhanced the antimicrobial effects. It was noted that the use of acetic acid as a rinse for beef tissue led to sublethal injury of bacterial cells. An increase in organic material, such as rumen fluid, dirt, or manure, resulted in less effective reduction of *S. Typhimurium*. Unda et al. (1991) inoculated beef roasts by injection and on the

surface with approximately 10^3 CFU of *Clostridium sporogenes* and *L. monocytogenes*. The acetic acid in the brine inhibited anaerobic and aerobic bacteria.

Intervention steps incorporated into poultry processing have been effective in reducing microbial contamination. Poultry scald water containing 0.1% acetic acid at 52°C decreased levels of *S. Typhimurium* and *Campylobacter jejuni* by 0.5 to 1.5 logs (Okrend et al., 1986). Addition of 1.0% acetic acid caused instantaneous death. Levels of 0.2% to 0.5% acetic acid reduced *Enterobacteriaceae* counts by 2.24 log (Lillard et al., 1987). Because of the possible attachment of *Salmonella* to broiler chicken skin, greater than a 4% concentration of acid was needed to reduce levels by 2 logs (Tamblyn and Conner, 1997). Despite these treatments, further poultry processing can reintroduce microorganisms onto carcasses.

Acidification of the chill water at the end of processing delivers a second intervention step to reduce microbial loads and to increase the shelf life of poultry. Adjustment of the chill water to pH 2.5 with acetic acid was the most effective antimicrobial treatment followed by other acids in descending order of effectiveness: adipic, succinic, citric, fumaric, and lactic (Mountney and O'Malley, 1965). Despite the reduction in microbial numbers at this pH, acetic acid caused the skin of the poultry to be hard and leathery.

Other studies continued to explore the use of acetic acid in chill water, but changes in pH and concentration of acid were more successful. Immersion of broilers for 10 minutes in 0.6% acetic solution acid (pH 3.0) did not result in a significant reduction of aerobic plate counts but did significantly reduce levels of Enterobacteriaceae by 0.71 log most probable number (MPN)/ml (Dickens et al., 1994). Expanding on their work, Dickens and Whittemore (1994) exposed broilers to 0.3% and 0.6% acetic acid under the same conditions, with and without the use of air injection to agitate the chill water. Again, aerobic plate counts were unaffected by the treatments, but Enterobacteriaceae counts were significantly reduced by 0.86 log MPN/ml for the 0.3% acid and 2.35 log MPN/ml for the 0.6% acid solutions. Air injection did not affect reduction of these counts. There was no significant difference in texture or sensory characteristics between the treatments, although the skin of the 0.6% acetic acid-treated carcasses was darkened or yellowed (Dickens et al., 1994). Water pockets occurred under the skin of chicken carcasses with air-agitated samples (Dickens and Whittemore, 1994).

Products are now being preserved by a number of antimicrobial compounds using a multiple-barrier approach to preservation. Kurita and Koike (1983) combined 3% ethanol, 0.05% acetic acid, 1 mM perillaldehyde—or 0.5%, 0.05%, and 0.5 mM, respectively, of the same components—with 2% salt to inhibit growth of contaminating microorganisms for more than 20 days at 27°C. Rubin (1978) found that acetic and lactic acid acted synergistically to retard growth of *S. Typhimurium* using a model system. El-Shenawy and Marth (1989b) investigated the effect of acetic, citric, lactic, and tartaric acid adjusted to pH 5.6 or 5.0 on inhibition of *L. monocytogenes*. Acidified media showed greater inhibitory activity at 35°C than at 13°C. Acetic and tartaric acids were more inhibitory than lactic and citric acids.

The acid level could be reduced when used in combination with brines and sugar. Whole pickles preserved with brine at 12° Brix and 0.9% acetic acid did not support growth or toxin formation of *Clostridium botulinum* (Ito et al., 1976). Stroup et al. (1985) found that mushrooms reached an equilibrium pH of 4.6 depending on the concentration of acid in can brine within 1 day with 1.0% acetic acid compared to 2 days for 0.7% citric acid. For pearl onions, a 0.35% solution required 7 days for equilibration for acetic and citric acid but 1 day for 0.7% acetic and 4 days for the same concentration of citric acid.

Nisin (an approved bacteriocin for some foods; see Chapter 7) coupled with 0.05% and 0.5% concentrations of acetic, lactic, or citric acids or glucono- δ -lactone were studied using *Bacillus* spores heated at pasteurization temperatures of 65°C and 95°C and recovered at 12°C, 20°C, and 30°C (Oscroft et al., 1990). Although each individual acid influenced the heat resistance of *Bacillus* spores differently, acetic acid was the most destructive acid at all combinations of parameters. As the incubation temperature to recover spores was lowered, germination and outgrowth of *Bacillus*

spores were more restricted. Nisin, in combination with organic acids, displayed synergistic effects in increasing the destruction of the organism. It was found that acetic acid was the most effective acid, followed by glucono-delta lactone, lactic, and citric acid when spores were heated at 95°C for 15 minutes and acetic, lactic, glucono-delta lactone, and citric acids when temperatures were reduced to 65°C for 60 minutes. Foods, particularly precooked, chilled, ready-to-eat products, are especially susceptible to spoilage because of indigenous microbial populations. A combination of these parameters could be used to extend the shelf life of these products.

Although acetic acid is generally used as an inhibitor of bacteria and yeasts, it is effective against the black bread molds, *A. niger*, and *Rhizopus nigrificans* at pH 3.5. *Aspergillus fumigatus* required 0.4% acetic acid at pH 5.8 and 0.2% acid at pH 5.0 and below to inhibit growth (Kirby et al., 1937). When used as a surface application, a 1% concentration of acetic acid at pH 4.5 completely inhibited growth and aflatoxin production by *Aspergillus parasiticus*. Lower concentrations of 0.6% or 0.8% partially inhibited growth and decreased toxin formation by 70% and 90%, respectively (Buchanan and Ayres, 1976). Cruess and Irish (1932) showed that apple juice containing 0.8% to 1.0% acetic acid adjusted to pH 3.5 inhibited *Saccharomyces ellipsoideus* and *Penicillium glaucum*, but a concentration greater than 4% was needed when the pH was at 7.0. Populations of *Pseudomonas aeruginosa* were reduced by 99.999% after exposure to a 1.0% concentration of acetic acid, suggesting its use as a decontamination agent for equipment (Hedberg and Miller, 1969).

Other forms of acetic acid have antimicrobial uses. Peracetic acid is formed by an oxygen molecule bound to the carboxyl carbon atom of acetic acid. On contact with organic substrates, it decomposes to yield oxygen and acetic acid. Spore-forming bacteria are killed on surfaces at a concentration of 0.0001 and 40% relative humidity within 10 minutes (Portner and Hoffman, 1968). A combination of peroxyacetic (64 ppm)/octanoic (53 ppm) acid solution was more effective as an antifungal agent in flume water for vegetables such as celery, cabbage, and potatoes than peroxyacetic (80 ppm) acid alone (Hilgren and Salverda, 2000). This treatment could also be used in recycling of water to reduce levels of yeasts and molds, aerobic bacteria, and coliforms.

Toxicology

Acute toxicity data for acetic acid in mice and rats indicate varied tolerance levels by different routes of administration (Table 4.2). Ingestion of acetic acid by humans has caused burned lips (Palmer, 1932), interference with blood coagulation (Fin'ko, 1969), pneumonia (Gerhartz, 1949), renal failure (Paar et al., 1968), gastric erosion (O'Keane et al., 1971), and death.

Rats fed 10% acetic acid and cats fed 20% acid developed ulcers in the gastric mucosa (Okabe et al., 1971). Johnson (1968) noted that rat gastric mucosa responded to a 100-mM concentration of acetic acid by releasing histamine into the interstitial fluid. Rats consuming acetic acid in drinking water in concentrations of 0.01% to 0.2% (0.2 g/kg body weight) did not show any adverse effects. At the 0.5% level, however, rats decreased their food intake level and lost 2.6% of their body weight (Sollman, 1921). Mori (1952) fed rats acetic acid ranging from 10 to 50 cm³ acid/kg of rice. All rats showed mucosa damage owing to the feeding method; however, it was not possible to determine the actual quantity of acid consumed.

Studies on nonmammalian species showed that acetic acid at a concentration of 0.0001 and 0.001 M accelerated spindle activity and retarded anaphase in the mitotic cycle of the root tips of the onion, *Allium cepa* (Makinen, 1958). Grasshoppers treated with 0.04 M acetic acid at pH 3 to 4 showed chromosomal and chromatid breaks. Damage from acetic acid resembled that from ionizing radiation or radiometric chemical damage (Manna and Mukherjee, 1966).

Rats exposed to vapor levels of acetic acid showed muscle imbalance at 0.2 or 5 mg/m³ but not at 0.01 mg/m³. Levels of 0.48 mg/m³ altered sensitivity to light, and 0.29 mg/m³ caused changes in electroencephalograms. Human olfactory thresholds to acetic acid vapor were 0.60 mg/m³. Takhirov (1969) suggested limits of 0.06 mg/m³. Parmeggiani and Sassi (1954) reported that workers

TABLE 4.2
Available Acute Toxicity Data (LD₅₀) for Various Acids

Acid	Animal	Route of Administration	LD ₅₀ (mg/kg body weight)	Reference	
Acetic	Mouse	Oral	4,960	Spector (1956)	
		Intravenous	525	Orö and Wretland (1961)	
	Rat	Oral	3,310–3,530	Spector (1956)	
Acetate, sodium	Mouse	Intravenous	380	Spector (1956)	
Dehydroacetic	Rat	Oral	1,000	Spencer et al. (1950)	
Adipic	Mouse	Oral	1,900	Horn et al. (1957)	
		Intravenous	680		
		Intraperitoneal	275		
	Rabbit	Oral	2,430–4,860	Enders (1941)	
		Intravenous	2,430		
Caprylic	Mouse	Intravenous	600	Orö and Wretland (1961)	
Citric	Mouse	Oral	5,040–5,790	Yokotani et al. (1971)	
		Intravenous	940–960		
		Intravenous	42		Gruber and Halbeisen (1948)
			Intravenous	203	Horn et al. (1957)
			Intraperitoneal	961	Gruber and Halbeisen (1948)
			Subcutaneous	2,700	Yokotani et al. (1971)
	Rat		Oral	11,700	
			Intraperitoneal	725	
			Intraperitoneal	884	Gruber and Halbeisen (1948)
			Subcutaneous	5,500	Yokotani et al. (1971)
Rabbit	Intravenous	330	Gruber and Halbeisen (1948)		
Citrate, sodium	Mouse	Intravenous	44	Gruber and Halbeisen (1948)	
		Intraperitoneal	1,460		
	Rat	Intraperitoneal	1,210		
	Rabbit	Intravenous	338		
Lactic	Rat	Oral	3,730	Spector (1956)	
	Guinea pig	Oral	1,810		
Propionic	Mouse	Intravenous	625	Orö and Wretland (1961)	
Propionate, calcium	Rat	Oral	3,340	Hara et al. (1963)	
		Intravenous	580–1,020		
Propionate, sodium	Rat	Oral	5,100	Hara et al. (1963)	
		Intravenous	1,380–3,200		
Tartaric	Mouse	Intravenous	485	Horn et al. (1957)	

exposed for 7 to 12 years to acetic acid vapors in the production of acetyl cellulose displayed corrosion of the hands, eyes, teeth, pharynx, and lungs with an air concentration of 0.2 to 0.65 mg/L. Men having 7 to 25 years of exposure displayed blood abnormalities (Cesaro and Granata, 1955). In rare instances acetic acid, as well as citric, lactic, and tartaric acids, caused allergic reactions by producing canker sores (Tuft and Ettelson, 1956), cold sensitivities (Wiseman and Adler, 1956), and epidermal reactions (Weil and Rogers, 1951).

No toxicologic data were available for peracetic acid, although it is assumed that this derivative would be assimilated in a manner similar to that for acetic acid.

TABLE 4.3
Acceptable Daily Intake for Humans

Acid	Limitations (mg/kg body weight)		Reference
	Unconditional	Conditional	
Acetic	Not limited		FAO (1965)
Acetate, Ca ⁺ , K ⁺ , Na ⁺	Not limited		FAO (1963, 1973)
Sodium diacetate	0–15		FAO (1973)
Adipic		0–5	FAO (1965)
Citric ^a	Not limited		FAO (1966)
Citrate, Ca ⁺ , K ⁺ , Na ⁺	Not limited		FAO (1963)
Fumaric	0–6		FAO (1974)
Lactic	Not limited		FAO (1965)
DL-lactic		0–100 ^b	FAO (1965)
Lactate, Ca ⁺ , K ⁺ , NH ₄ ⁺ , Na ⁺	Not limited		FAO (1973)
Malic	Not limited		FAO (1965)
DL-malic		0–100 ^c	FAO (1966)
Propionic	Not limited		FAO (1965)
Propionate, Ca ⁺ , K ⁺ , Na ⁺	Not limited		FAO (1973)
Tartaric	0–30		FAO (1973)
Tartrate, K ⁺ , Na ⁺	0–30		FAO (1973)

^a Naturally occurring substances; the estimated acceptable daily intakes listed here do not include amounts occurring naturally.

^b Content of DL-lactic acid.

^c Content of DL-malic acid; the malic acid content of malic acid should not exceed 0.05%.

Application and Regulatory Status

Acetic acid is a monocarboxylic acid with a pungent odor and taste, which limits its use. It is the principal component of vinegars and as such is primarily used for its flavoring abilities. It is highly soluble in water. It is used in condiments such as mustard, catsup, salad dressings, and mayonnaise and is found in pickled products such as sausages and pigs' feet. Because of cost and antimicrobial action, it has been added to infant feeding formulas to replace lactic acid (Seymour et al., 1954). Acetic acid is generally regarded as safe (GRAS) for miscellaneous and general-purpose usage (21 CFR 184.1005). The acceptable daily intakes for humans for acetic acid and its derivatives are listed in Table 4.3. The data are scarce for the use of peracetic acid in food products. It has primarily been used as a surface disinfectant and is used for preventing fruit flies from laying eggs in damaged tomatoes (Ayres et al., 1980).

ACETATES

Antimicrobial Properties

Several derivatives of acetic acid are currently in use as antimicrobial agents. The sodium (SA) and calcium salts are sometimes used in foods and would be expected to have the same antimicrobial properties as acetic acid at the same pH values (Hoffman et al., 1939). The salt form, however, requires different handling and use procedures than the acid. Mendonca et al. (1989a) used a dip of SA in combination with potassium sorbate (10%) and phosphates (10%) to extend the shelf life of pork chops.

SA (10%), potassium sorbate (1.5%), sodium lactate (3%), or trisodium citrate were used alone or in combination with *Bifidobacterium breve* (5%) as a dip for fresh camel meat (Al-Sheddy et al., 1999). SA extended shelf life to 12 days at 4°C, whereas potassium sorbate increased shelf life to 9 days. *B. breve*, sodium lactate, and trisodium citrate extended shelf life to 6 days. A combination of *B. breve* and SA exhibited an additive effect that extended shelf life still further. Color remained acceptable, although a mild acetic acid odor was detected.

SA (0.5%) was also effective in reducing populations of *L. monocytogenes* in vacuum-packaged turkey bologna during storage at 4°C and was similar to 0.26% potassium sorbate or 2% sodium citrate in its efficacy (Wederquist et al., 1994). A combination of 0.25% acetate and 2.5% lactate was effective as an antimicrobial treatment in sliced serelat sausage over a 4- to 6-week storage life (Blom et al., 1997).

SA at concentrations of 0.75% and 1% significantly reduced the initial aerobic plate counts of catfish fillets by 0.6 to 0.7 logs when stored at 4°C compared with the nontreated fillets and increased shelf life by 6 days. The combination of 0.3% to 0.7% SA and 0.4% monopotassium phosphate (MKP) more effectively prolonged shelf life to 12 days compared with SA alone, although MKP alone had no effect. SA alone or SA-MKP-treated fillets were not significantly different in odor from fresh controls up to 9 days, but they did appear brownish and watery (Kim et al., 1995b). SA in combination with potassium sorbate and *Lactococcus lactis* ssp. *cremoris* culture added to catfish fillets was an effective treatment against growth of Gram-negative spoilage bacteria at 4°C (Kim and Hearnberger, 1994). The combination of 0.5% SA, 0.25% potassium sorbate, and 2.5% lactic cultures or 0.5 to 1.0% SA alone inhibited growth for more than 6 days at 4°C and counts increased only by 1.3 or 1.2 logs by day 12. Treatment with SA alone or in combination with bifidobacteria extended shelf life at 4°C by 3 days (Kim et al., 1995a). Catfish fillets treated with 2% SA, vacuum packaged, and held at 4°C for 12 days contained lower population levels of psychrotrophic and anaerobes than untreated samples (Zhuang et al., 1996).

Whole and peeled shrimp were immersed for 2 minutes in solutions containing 10% SA, 1.5% potassium sorbate, 5% sodium lactate, and 1.5% trisodium citrate with or without *B. breve* culture and stored at 4°C for 9 days (Al-Dagal and Bazaraa, 1999). Psychrotrophic counts from untreated whole shrimp increased to $>10^7$ /g, the indicator of spoilage, over 6 days, but bifidobacteria did not grow within 9 days; SA alone or potassium sorbate with bifidobacteria retarded growth of psychrotrophs compared with other treatments within 6 days and provided the lowest counts with the potassium sorbate-bifidobacteria mixture. For peeled shrimp, untreated samples reached the spoilage mark within 10 days, but SA provided better sensory characteristics and more controlled growth of psychrotrophs than potassium sorbate with bifidobacteria. Growth and aflatoxin production of *A. parasiticus* was inhibited by a 1.0% concentration of SA at pH 4.5. At lower acid concentrations of 0.6% or 0.8%, growth and toxin formation were decreased 70% and 90%, respectively (Buchanan and Ayres, 1976).

Toxicology

The LD₅₀ (mean lethal dose) for SA in mice is presented in Table 4.2. Chickens fed diets supplemented with 5.44% SA showed decreased appetite, depressed growth rate, and increased mortality. Histologic findings showed hypertrophy of the kidneys and ureters. Waterhouse and Scott (1962) attributed the toxic effect to the sodium ion rather than the acid moiety. A 22-year-old woman with sensitivity to acetic anhydride was not allergic to SA (Weil and Rogers, 1951). No toxicologic data were available for calcium acetate (Food and Agricultural Organization, 1963), although it is assumed that this derivative would be assimilated in a manner similar to that for acetic acid.

Application and Regulatory Status

Sodium (21 CFR 184.1721) and calcium (21 CFR 184.1185) acetates are approved as GRAS substances for miscellaneous and general-purpose usage. SA can be used as a boiler additive for food-grade steam (Federal Register, 1962). Calcium acetate is classified as a stabilizer when migrating from food packaging materials (21 CFR 181.29).

SODIUM DIACETATE

Antimicrobial Properties

Sodium diacetate was effective at a 0.5% level in malt syrups. Levels of 0.1% to 2.0% delayed mold growth in cheese spreads, and when it was incorporated into butter wrappers, it prevented mold growth (Chichester and Tanner, 1972). Sodium diacetate is equally effective in the baking industry where its inhibitory powers prevent the growth of bread mold and rope-forming bacteria, such as *Bacillus mesentericus (subtilis)*, while having little effect on baker's yeast, *Saccharomyces* species (Glabe and Maryanski, 1981). A concentration of 0.05% to 0.4% at pH 3.5 inhibited *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Aspergillus glaucus*, and *Penicillium expansum*; a concentration of 0.15% to 0.5% at pH 4.5 inhibited these same strains and *Mucor pusillus* in feeds and silage.

The minimum inhibitory concentration (MIC) of sodium diacetate was determined for *L. monocytogenes* grown in BHI broth adjusted to pH 5.25, 5.4, 5.55, 6.0, and 6.3 and incubated at 5°C, 20°C, and 35°C (Shelef and Addala, 1994). An interactive effect was noted between temperature and concentration of acid in that growth was inhibited with decreasing concentrations of acid and temperatures. The MIC was determined to be 35 mM (pH 5.25) at 35°C, 32 mM (pH 5.4) at 20°C, and 28 mM (pH 5.55) at 5°C. Sodium diacetate was more effective than acetic acid, and the inhibitory effect was attributed to the diacetate moiety rather than pH alone.

Cooked-in-bag ham was formulated with additions of 0.1% or 0.2% sodium diacetate, 2.5% or 3.3% sodium lactate, or 1% buffered sodium citrate (Stekelenburg and Kant-Muermans 2001). *Lactobacillus curvatus* and *L. monocytogenes* were inoculated into the cooked ham products at an initial level of 10^4 and 10^2 /g of product, vacuum-packaged, and stored for up to 40 days at 4°C. *L. curvatus* reached an end point of 10^7 /g within 2 to 2.5 weeks in products containing 0.1% and 0.2% sodium diacetate or 1% buffered sodium citrate and within 3 and 5 weeks for products containing 2.5% and 3.3% sodium lactate, respectively. *L. monocytogenes* was inhibited by sodium lactate and 0.2% sodium diacetate. However, in products containing buffered sodium citrate, *L. monocytogenes* increased to approximately 10^6 /g within 3 weeks. The addition of sodium diacetate affected the sensory qualities of the product precluding its use. In ground beef, the addition of 0.3% sodium diacetate was effective in suppressing total aerobic counts. In addition to *L. monocytogenes*, sodium diacetate was also effective against hemorrhagic *E. coli*, *Pseudomonas fluorescens*, *S. Enteritidis*, *Shewanella putrefaciens*, and *B. cereus* with no effect against *Pseudomonas fragi*, *Y. enterocolitica*, *Enterococcus faecalis*, *Lactobacillus fermentans*, or *S. aureus* (Shelef and Addala, 1994).

Sodium diacetate at concentrations of 0.1% to 0.3% and sodium lactate at concentrations of 2% to 3% were equally effective as antilisterial compounds in meat with little effect on pH and sensory characteristics (Mbandi and Shelef, 2001). *L. monocytogenes* and *S. Enteritidis* were inoculated into a comminuted beef emulsion formulated with 0.1% and 0.2% sodium diacetate, 0.2% SA, or 1.8% and 2.5% sodium lactate or combinations of the acids and held at 5°C or 10°C. The growth of *L. monocytogenes* was not significantly different in the presence of the individual inhibitors compared with control samples after 20 days at 10°C. However, the combination 0.2% sodium diacetate and 2.5% sodium lactate was bacteriostatic to *L. monocytogenes* and bactericidal

to *S. Enteritidis*. At lower temperatures, lower concentrations of the antimicrobials were equally as effective. Sodium lactate did not affect the pH of the meat, but sodium diacetate reduced the pH slightly. In subsequent work (Mbandi and Shelef, 2002), the reduction of multiple strains of *L. monocytogenes* and *Salmonella* species was demonstrated using 2.5% sodium lactate and 0.2% sodium diacetate alone or in combination in beef bologna, a more complex product, stored aerobically at 5 and 10°C. As expected, the combination of acids achieved greater reductions of both organisms at both temperatures compared to individual acids and greatly reduced background spoilage microflora.

A surface application of 1 to 3 mg/cm² (2 to 6 g/chicken) sodium diacetate powder extended the shelf life of chickens about 4 days when held at 2°C. The powder dissolved in the surface moisture of the carcass to form acetic acid and SA and produced a surface pH of about 4.8. Enterobacteriaceae counts dropped 1000-fold over 6 days at 2°C. Sensory characteristics of roasted chickens were not affected even at levels up to 8 g per chicken (Moye and Chambers, 1991).

Schlyter et al. (1993b) used the multiple-barrier concept to limit growth of *L. monocytogenes* in turkey mixtures. Slurries of uncured turkey breast, prepared with sodium diacetate (0.1%, 0.3%, 0.5%), sodium lactate (2.5%), and pediocin (5000 U/ml), were pasteurized for 10 minutes at 68°C after which they were inoculated with *L. monocytogenes* at a level of approximately 4.5 logs CFU/ml slurry. Mixtures were incubated at 25°C or 4°C and sampled up to 7 and 42 days, respectively. Sodium diacetate was determined to be listericidal over the 7-day period at 25°C only at the 0.5% concentration (0.54 log reduction). *L. monocytogenes* in mixtures held at 4°C for 42 days was inhibited at 0.3% and 0.5% concentrations (0.59 log reduction). Lactate and pediocin enhanced the listericidal properties of diacetate suggesting some type of synergistic activity. Sodium diacetate (0.5%) was listericidal in combination with ALTA™, a fermentation by-product of lactic acid bacteria, producing a 2-log reduction after 7 days at 25°C (Schlyter et al., 1993a).

The growth of *L. monocytogenes* was reduced by 2.6 logs in crabmeat stored at 4°C for 6 days when treated with 2 M sodium diacetate. Although 1 M sodium lactate solution reduced populations within 2 days, populations increased approximately 0.5 logs within 6 days. Application of 4 M SA reduced the levels of *L. monocytogenes* by 0.8 logs (Degnan et al., 1994).

Toxicology

No toxicologic data were available for sodium diacetate (Food and Agriculture Organization, 1962), although it is assumed that this derivative would be assimilated in a manner similar to that for acetic acid.

Application and Regulatory Status

Sodium diacetate is approved as a GRAS substance for miscellaneous and general-purpose usage (21 CFR 184.1754). It is used in cheese spread, malt syrups, butter, and wrapping material. In bread and cake products, use levels range from 0.25% to 0.4% (Glabe and Maryanski, 1981).

DEHYDROACETIC ACID

Antimicrobial Properties

Dehydroacetic acid (DHA) has one of the highest dissociation constants (Table 4.1) of the acidulants and remains effective at higher pH ranges. This acid is effective against *Enterobacter aerogenes* and *L. plantarum* at levels of 0.3% and 0.1%, respectively, although it is fungistatic at concentrations of 0.005% to 0.1% (Wolf, 1950). Sodium dehydroacetate was twice as effective as sodium benzoate at pH 5.0 against *S. cerevisiae* and 25 times more effective against *P. glaucum* and *A. niger* (Banwart, 1989).

Strawberries dipped in or sprayed with 0.25% or 5% sodium dehydroacetate for 60 seconds were considered more marketable after 3 days of storage at 20°C than water-dipped berries. Furthermore, treatment with sodium dehydroacetate was effective in reducing respiration rate and delaying ripening, thus extending marketability (Watada, 1971).

The effects of potassium sorbate, sodium *p*-hydroxybenzoate, benzoic acid, and sodium dehydroacetate acid were investigated for three strains of *E. coli* O157:H7 in broth and model meat products. The MIC in broth at pH 6.5 was 4 mg/ml for potassium sorbate, benzoic acid, and sodium dehydroacetate and 16 mg/ml for sodium *p*-hydroxybenzoate. Sorbate was bacteriostatic at half the MIC for *E. coli* in homogenized beef but not in poultry (Yamamura et al., 2000).

Toxicology

The LD₅₀ for DHA in rats is given in Table 4.2. Spencer et al. (1950) did not show any unusual effects in rats receiving 10, 30, or 100 mg/kg of DHA per day for 34 days. However, 300 mg/kg led to severe weight loss and damage to internal organs. A 2-year feeding study with male and female rats fed 0.02%, 0.05%, and 0.1% of the substance showed no changes in growth, mortality, appearance, hematology, or histopathology. No toxic effects were noted in monkeys given doses of 50 and 100 mg/kg at a rate of five times per week for 1 year, although a dose of 200 mg/kg led to growth and organ changes. The sodium salt of DHA was nonirritating to rabbit's skin, even with prolonged contact (Spencer et al., 1950).

Application and Regulatory Status

DHA or its sodium salt is GRAS when used in accordance with good manufacturing practices. It is approved for use as a treatment for cut or peeled squash in amounts equal to or less than 65 ppm remaining in or on prepared squash (21 CFR 172.130). It can also be used as a fungistat in cheese wrappers.

ADIPIC ACID

Antimicrobial Properties

Adipic acid has no antimicrobial properties other than those ascribed to its ability to reduce pH. This acid at the appropriate concentration level would inhibit the growth of any microorganisms susceptible to low pH values.

Toxicology

Acute toxicity data for adipic acid in mice and rabbits indicated varied tolerance levels by different routes of administration (Table 4.2). Oral administration of the acid to mice caused hemorrhaging in the small intestine with distension of the stomach. Intravenous and intraperitoneal injection produced hemorrhaging in the lungs, in addition to acidosis by the former route and intestinal adhesions by the latter route (Horn et al., 1957). Enders (1941) showed similar symptoms in rabbits, which included marked obstruction in the liver and kidneys by the oral route and polyuria by intravenous administration, resulting in the loss of up to 20% of body weight.

Adipic acid did not affect female rats fed at levels of up to 40 mg/day or male rats fed up to 800 mg/day in short-term studies (Lang and Bartsch, 1953). However, the 800 mg/day dosage depressed growth, produced diarrhea, altered behavior, and caused slight histologic aberrations. Longer studies using male and female rats given 400 or 800 mg/day did not affect offspring. However, at the higher dosage levels, weight gains were lower and mortality was higher than in the controls (Lang and Bartsch, 1953). The 90-day feeding trials conducted on rats fed 0.1%, 1.0%, or 5.0% adipic acid showed no effect at the two lowest levels, but significant growth depression

occurred at the 5.0% level although the food intake level was the same. Sodium adipate at the 5% level decreased the rate of weight gain (Informatics, Inc., 1943).

The 2-year feeding trials using male rats fed 0.1%, 1.0%, 3.0%, and 5% adipic acid and female rats fed 1.0% acid did not show any abnormalities; however, weight gains for rats fed the two highest levels were significantly lower than those for control rats (Horn et al., 1957).

Teratologic studies using mice, rats, and hamsters showed no significant difference from controls at levels of 263, 288, and 205 mg/kg body weight, respectively (Food and Drug Research Laboratory, 1973b). Inhalation of adipic acid for 6 hours at a rate of 126 µg/l produced no unusual findings (Gage, 1970).

Application and Regulatory Status

Adipic acid is a nonhygroscopic, saturated dicarboxylic acid having low water solubility. It is four to five times more soluble than fumaric acid at room temperature and has the lowest acidity of any of the food acids. It is used in gelatin desserts, baking powders, powdered fruit beverages, candies, refrigerated rolls, and biscuits because it imparts a smooth, mildly acid flavor. It also serves a function in the canning of vegetables, as a sequestrant in oils, and in improving the melting characteristics of processed cheese (Gardner, 1972). Adipic acid is approved as a GRAS substance when used as a buffer or neutralizing agent not to exceed good manufacturing practices (21 CFR 184.1009). The acceptable daily intake for humans is listed in Table 4.3.

CAPRYLIC ACID

Antimicrobial Properties

When tested against a variety of Gram-positive and Gram-negative organisms and yeasts, caprylic acid was ineffective at concentrations of up to 7.8 µmol/ml (Kabara et al., 1972). At pH 6.0, caprylic acid inhibited bacteria and fungi at a lower concentration than either acetic or propionic acid. At pH 5.0, however, the inhibitory concentrations were not dramatically lower (Woolford, 1975b). The MIC of caprylic acid was 100 µg/ml in TSB for *Vibrio parahaemolyticus* (Beuchat, 1980). Caprylic acid at a 0.3% concentration inhibited *E. coli* when grown in a chemically defined medium and was more effective than 1.5% acetic acid and 1.8% propionic acid. Caprylic acid was also more inhibitory to *Shigella* species than either acetic or propionic acids (Nakamura and Zangar, 1968).

Toxicology

Orö and Wretland (1961) determined the LD₅₀ of caprylic acid for mice by intravenous administration (Table 4.2).

Application and Regulatory Status

Caprylic acid is a colorless, oily liquid that has a slightly unpleasant odor and a burning, rancid taste. It is slightly soluble in water. It is used as a flavoring adjuvant in levels not to exceed 0.013% for baked goods; 0.04% for cheese; 0.005% for fats and oils, frozen dairy desserts, gelatins and puddings, meat products, and soft candy; 0.16% for snack foods; and 0.001% or less for other food categories. Caprylic acid is approved as a GRAS substance for miscellaneous and general-purpose usage (21 CFR 184.1025). Caprylic acid is also used as an antimicrobial agent for indirect use in cheese wraps, not to be used in amounts exceeding good manufacturing practice. No limit has been set on the acceptable daily intake for humans.

CITRIC ACID

Antimicrobial Properties

Inhibition of *L. monocytogenes* was achieved in trypticase soy yeast extract broth (TSBYE) adjusted to pH 5.0 with propionic acid; 4.5 with acetic and lactic acids; and 4.0 with citric and hydrochloric acids (Conner et al., 1990). The effect was temperature dependent in that survival of *L. monocytogenes* decreased to undetectable levels within 1 to 3 weeks at 30°C, whereas at 10°C, *L. monocytogenes* survived after 11 to 12 weeks in TSBYE adjusted with acetic, citric, and propionic acids and for 6 weeks in media containing HCl or lactic acid. Similar pH-temperature relationships were noted by Cole et al. (1990), who determined that the minimum pH values that allowed survival of *L. monocytogenes* after 4 weeks were pH 4.66 at 30°C, 4.36 at 10°C, and 4.19 at 5°C. The minimum pH that permitted growth within 60 days (>100-fold increase in cell numbers) was the same at 30°C but increased to 4.83 at 10°C and 7.0 at 5°C. These experiments used citrate phosphate buffer in which the citric acid content was calculated at 1.1% at pH 4.4 and 0.7% at pH 6.0. The authors suggested the inhibitory activity might be the result of the chelating ability of citric acid. Additional interactive effects were also noted in the presence of salt. Sorrells et al. (1989) reported that on an equal molar basis, citric acid was more inhibitory than lactic acid, followed by acetic acid. Interaction was demonstrated between citric acid and sodium citrate added to BHI broth adjusted to pH 4, 5, 6, and 7 (Buchanan and Golden, 1994). Rate of inactivation of *L. monocytogenes* after exposure to the acid combinations was dependent on pH and acid concentration whereby pH 5 and 6 appeared to be protective against inactivation, but lower pH levels were toxic.

The inhibitory capacity of hydrochloric, citric, acetic, lactic, propionic, and phosphoric acid was compared in TSB for *Y. enterocolitica* based on concentration of acid, pH, and degree of dissociation. A comparison of equimolar concentrations identified citric acid as the most antimicrobial, followed by hydrochloric, lactic, phosphoric, propionic, and acetic. Based on pH, the highest inhibitory activity was associated with propionic, then lactic, acetic, citric, phosphoric, and hydrochloric. The activity of the undissociated portion of the acid was hydrochloric followed by lactic propionic, and acetic (Brackett, 1987).

Citric acid at a concentration of 0.5% (pH 4.5) was more effective than lactic acid (pH 3.4) in inhibiting *Arcobacter butzleri* in a broth system (Phillips, 1999). Sodium citrate was also more effective than 2% sodium lactate, and the combination of 1.5% sodium citrate and 1.5% sodium lactate was equally inhibitory as sodium citrate alone, indicating that citrate was the more effective compound. Citric acid was more inhibitory to thermophilic bacteria than acetic or lactic acids; however, pH was not considered a reliable indicator of preservation effectiveness (Fabian and Wadsworth, 1939; Fabian and Graham, 1953). Citric acid was particularly inhibitory to flat-sour organisms isolated from tomato juice. Little bacteriostatic activity occurred at pH 5.0, but inhibition increased with decreasing pH (Murdock, 1950). When tomato juice was adjusted with citric acid to pH 4.0 and 3.7 and stored for 7 days at 5°C, lower aerobic plate counts of 3.8 and 2.6 log/ml were seen, respectively, compared to counts in unacidified juice (6.22 log/ml); aerobic counts for pH 3.7 juice incubated at 20°C were 6.12 log/ml. Yeast and mold counts were the reverse with counts of 2.09, 2.13, and 1.64 log/ml, respectively; aerobic counts at 20°C for pH 3.7 juice were 4.42 log/ml (Bizri and Wahem, 1994).

Acidification of foods before canning is often used to reduce the thermal process time of foods that are particularly sensitive to changes in sensory qualities, such as texture or appearance. Canned tomatoes acidified with citric, fumaric, or malic acids did not change in physical or chemical attributes during processing compared with unacidified tomatoes (Schoenemann and Lopez, 1973; Schoenemann et al. (1974). Okra, canned in a brine containing acetic, citric, lactic, malic, or tartaric acid to achieve an equilibrium pH of 4.3, was processed for 30 minutes in boiling water. Acidification impaired the color but enhanced the flavor of canned okra. All acids would be effective antibotulinal agents at that pH level (Nogueira et al., 1997).

Beelman et al. (1989) developed an acid-blanch-chelate (ABC) process designed to reduce mesophilic and thermophilic bacterial spoilage in canned mushrooms. They found that acid blanching in a citric acid (0.05 M) solution buffered to pH 3.5 followed by canning in a solution containing 200 ppm ethylenediamine tetraacetic acid (EDTA) controlled the outgrowth of spores of *C. sporogenes* PA3679 after a sublethal heating process (Okereke et al., 1990a). By adding citric acid to can brine, this process reduced thermophilic spoilage from 68% to 23.9% and spoilage was further reduced to 16.8% with the addition of EDTA. Only 2.4% of cans containing mushrooms vacuum hydrated by the ABC process spoiled (Beelman et al., 1989; Okereke et al., 1990b). This process improved quality and increased the microbial stability of canned mushrooms (Okereke and Beelman, 1990).

Mushroom extract acidified with citric acid to pH 6.7 (control), 6.22, 5.34, and 4.65 and asparagus with added citric acid or glucono-delta-lactone to pH levels of 5.9, 5.4, 5.1, 4.8, and 4.5 were inoculated with *C. sporogenes* PA 3679 spores. Extracts were then heated at 110°C, 115°C, 118°C, and 121°C. Although the heat resistance of *C. sporogenes* decreased with decreasing pH levels, heat resistance was not affected by higher processing temperatures, leading the authors to conclude that the extracts have a protective effect on *C. sporogenes*, thereby leading to its survival regardless of the change in pH (Ocio et al., 1994; Silla Santos et al., 1992).

Reduction in the thermal process time of canned liver paste was achieved through the addition of 0.14% citric acid (CA), 0.29% CA, 0.31% CA–2.0% trisodium citrate (TCA), or 0.31% CA–2.0% TCA–0.1% potassium sorbate (Houben and Krol, 1991). Liver pastes inoculated with 10^6 to 10^7 *Bacillus* spores per g and 10^4 *C. sporogenes* spores per g were heated to an F_0 of 0.05, 0.3, and 0.85. Microbial stability was achieved in liver paste formulated with 0.14% CA (pH 5.69) and processed with an F_0 of 0.3. The addition of potassium sorbate did not enhance stability.

Growth curves developed for *B. cereus* strains grown in model vegetable broths were conducted at typical refrigeration and abuse temperatures of 5°C, 8°C, 12°C, and 16°C (Valero et al., 2000; 2003). *B. cereus* grew in zucchini broth (pH 6.5) at 12°C but did not grow in broth acidified to pH 5.0 at temperatures below 16°C. Growth in carrot puree was temperature dependent because *B. cereus* could grow at pH 5.1 and 5.2, provided temperatures were at 16°C, but could grow at 8°C only when the pH was higher (5.4 and 5.5), indicating a synergistic effect between pH and temperature. Both vegetable broths stimulated germination and growth of spores compared to nutrient broth.

Citric acid dips for foods not only retard some spoilage but also act as a chelator of metal ions responsible for enzymatic browning reactions. Cabbage and carrots were pretreated with the addition of a reduced calorie dressing or with a dip of 0.2% or 1.0% citric acid for 5 or 30 minutes. The products were packed with or without modified atmosphere and stored at 4°C for 10 to 21 days. Samples pretreated with 1% citric acid displayed significantly lower total numbers, coliforms, and lactic acid bacteria counts than samples dipped with the lower concentration of citric acid. Samples containing the reduced calorie dressing were even lower in microbial numbers than acid-dipped vegetables (Eytan et al., 1992). Notermans et al. (1985) dipped potatoes in a solution of 1% citric acid and 2% ascorbic acid for 2 minutes before vacuum packing and cooking. This process inhibited *C. botulinum* type B when held for 70 days at 15°C or 14 days at 20°C. Citric acid dips (0.3 g/kg) for crawfish (*Procambarus clarkii*) stored at 4°C were not effective in retarding growth of *L. monocytogenes* (Dorsa et al., 1993).

Homemade mayonnaise has been a significant health hazard as a result of contamination by *Salmonella* from raw eggs (Membre et al., 1997; Xiong et al., 1999). The preparation of these products included adjustment of the pH with acetic acid to 3.4 to 4.1 with holding temperatures between 18°C and 20°C for up to 3 days before consumption. Mayonnaise made with >5% citric acid solution adjusted to pH below 4.05 led to more inactivation of *S. Enteritidis* PT4 at 22 than 5°C, but the shelf life was shortened at this higher temperature. The authors recommended that the final pH should be <3.3 when at least 20 ml of lemon juice (citric acid) per fresh egg yolk was used. If the amount of lemon juice was increased to 20 to 35 ml per egg yolk, the product should

be held at 22°C for longer than 72 hours; when 35 ml lemon juice per egg yolk was added, the time decreased to 48 hours before use. Radford and Board (1993) found that acetic acid was more inhibitory than citric acid and recommended that mayonnaise be prepared with vinegar to a pH of 4.1 or less. The addition of garlic or mustard increased the death of *S. Enteritidis*, but salt had a protective effect on this organism.

C. botulinum showed no change in numbers when inoculated in tomato puree, shrimp puree, or shrimp and tomato sauce acidified with citric or acetic acids to pH 4.2 or 4.6. No toxin was detected in any of these foods over an 8-week shelf life (Post et al., 1985). Tsang et al. (1985) found no outgrowth or toxin production from spores of types A and B in TPGY medium acidified to pH 4.6 and incubated at 35°C; however, type E grew and produced toxin at 26°C in media containing citric acid at pH 4.2. When acetic acid was used, no growth occurred at pH 5.0.

Skim milk acidified with citric acid was more inhibitory to *S. Typhimurium* than lactic and hydrochloric acids (Subramanian and Marth, 1968). As little as 0.3% citric acid lowered the level of *Salmonellae* on poultry carcasses, and rinsing with a citric acid solution adjusted to pH 4.0 reduced the numbers of *P. fluorescens* on beef carcasses (Thomson et al., 1967); however, it did not appear to affect attachment (Appl and Marshall, 1984).

Mangoes are subject to spoilage through infection with *Penicillium cyclopium* (Palejwala et al., 1984). Sabouraud medium was adjusted with 0.125% to 4% citric acid or 0.016% to 1% malic acid as the sole carbon source because these acids predominate at these concentrations in a ripening mango. Healthy tissue had a pH of 5.8, whereas infected tissue was around 4.8. During ripening, the level of acidity decreased and sugars and nitrogenous compounds increased, leading to enhanced growth.

Ascospores of heat-resistant molds are difficult to kill during processing of fruit products and an increase in processing temperature can lead to adverse changes in quality (Rajashekhara et al., 1998). Survival studies for *Neosartorya fischeri* ascospores were conducted in mango and grape juices and heated at 75°C, 80°C, 85°C, and 90°C. Ascospores were able to withstand more than 6 hours of heating at 75°C, 5 hours at 80°C, and 3 to 4 hours at 85°C. Thermal resistance studies were conducted in mango juice adjusted to pH 3.5 with citric, lactic, malic, or tartaric acid. Citric acid provided the maximal destruction of ascospores at 85°C. Similar destruction values were seen with potassium sorbate and sodium benzoate. A level of 0.75% for both citric and lactic acids had a slight growth-retarding effect on *A. parasiticus*; a 0.25% concentration greatly reduced toxin production. A level of 0.5% citric or 0.75% lactic acid inhibited growth of *Aspergillus versicolor*; but 0.25% citric acid and 0.5% lactic acid prevented toxin production. A 0.75% concentration of either acid did not affect the growth of *P. expansum* (Reiss, 1976).

Sodium citrate can also exhibit bacteriostatic activity. Sodium citrate in concentrations of 0.1% to 4.0% was not as inhibitory to *Streptococcus agalactiae* when added to skim milk or fresh milk as citric acid at concentrations of 1%, 2%, and 4%. The MIC of 0.8% gave a pH of 4.08 to 4.12 (Sinha et al., 1968). Concentrations of 12.0% to 12.5% sodium citrate were inhibitory to *Salmonella anatum* and *Salmonella oranienburg* (Davis and Barnes, 1952).

S. aureus showed inhibition both with increasing concentrations of citric acid and decreasing pH (Minor and Marth, 1970). Citrate inhibition of *S. aureus* was overcome by adding calcium and magnesium ions, suggesting that citrate was a chelator of ions essential for growth (Rammell, 1962). The amount of sodium citrate had a dual effect on *Lactobacillus casei*. In concentrations of 12 to 18 µM/ml, sodium citrate stimulated growth, and in concentrations greater than 40 µM/ml, sodium citrate inhibited growth. Branen and Keenan (1970) also suggested that citrate chelation of metal ions inhibited *L. casei*. The metabolic effects of citrate on *Arthrobacter* species were twofold. With *Arthrobacter pascens*, the function of citrate appeared to be one of chelation of metal ions, even in media containing up to 1% citrate. However, with *Arthrobacter simplex*, citrate appeared to inhibit glucose utilization (Imai et al., 1970).

Using the multiple-barrier concept, hard-cooked eggs allowed to equilibrate in 0.5%, 0.75%, or 1.0% concentrations of citric acid and 0.2% sodium benzoate were held for 30 days at 4°C or

in 0.75% citric acid alone for 21 days at 4°C. A concentration of 0.75% citric acid was sufficient to reduce inoculated populations of *S. Typhimurium*, *Y. enterocolitica*, *E. coli*, and *S. aureus* (Fischer et al., 1985). Daly et al. (1973) used a combination of 0.1% citric acid and 0.75% glucono-delta-lactone incorporated into sausage before fermentation. This combination inhibited the growth of *S. aureus* early in the fermentation and the increased acidity of the meat mixture allowed the fermentation to proceed more quickly. Restaino et al. (1982) used a combination of 0.05% potassium sorbate and citric acid at pH 5.0 to reduce the growth rate of *S. rouxii* and *Saccharomyces bisporus*. Potassium sorbate at 0.2% concentration in combination with citric or lactic acid at pH 5.5 depressed growth of *L. plantarum* but did not affect *P. aeruginosa* (Restaino et al., 1981).

Toxicology

Acute toxicity data for citric acid in mice, rats, and rabbits indicated varied tolerance levels by different routes of administration (Table 4.2). Studies by Yokotani and coworkers (1971) compared toxicities of commercial citric acid and Takeda citric acid, a by-product of yeast fermentation. Death by all routes and in all animals resulted from respiratory or cardiac failure and hemorrhaging of the gastric mucosa. Gruber and Halbeisen (1948) used comparable levels of acid but suggested that many of the symptoms produced by high levels of citric acid resembled those of calcium deficiency. Horn et al. (1957) evaluated dosage levels in mice given the acid intravenously. Deaths resulted from acute acidosis and lung hemorrhages. Short-term studies on rats fed 0.2%, 2.4%, and 4.8% citric acid in a commercial diet showed lowered weight gains as a result of decreased intake of food and minor blood chemistry abnormalities at the 4.8% level; slight atrophy occurred in regions of the thymus and spleen (Yokotani et al., 1971).

Long-term studies involving rats fed quantities up to 1.2%, 3%, or 5% citric acid (Bonting and Jansen, 1956; Horn et al., 1957) showed no abnormalities other than lowered weight gains and feed consumption in the 5% group. Cramer et al. (1956) fed vitamin D-free diets containing low calcium levels but adequate levels of phosphorus supplemented with 0.02 mol sodium citrate and citric acid. Although there was no effect on weight gain, the urine contained calcium citrate. The authors concluded that citrate had a rachitogenic effect on the test animals. Dalderup (1960) fed rats 1.5, 4.5, or 12 g citric acid per kg of a noncariogenic diet to show the possible effect of citric acid on the development of teeth. The number of cavities formed did not differ between the control and experimental groups; however, the highest dosage contributed to enamel erosion.

Acute toxicity data of sodium citrate are shown in Table 4.2. Subcutaneous injections of 320 to 1200 mg/kg body weight of sodium citrate into dogs decreased blood calcium levels while increasing calcium levels in urine (Gomori and Gulyas, 1944). In another study, 7.7% sodium citrate (5% citric acid) fed to rabbits for 60 days produced no unusual effects (Packman et al., 1963).

Application and Regulatory Status

Citric acid is a tricarboxylic acid having a pleasant sour taste. It is highly water soluble and enhances the flavor of citrus-based foods. It is approved for use in ice cream, sherbets and ices, beverages, salad dressings, fruit preserves, and jams and jellies, and it is used as an acidulant in canned vegetables and dairy products. It is a precursor of diacetyl and therefore indirectly improves the flavor and aroma of a variety of cultured dairy products. It can control the pH for optimum gel formation. Citric acid also acts synergistically with antioxidants to prevent rancidity by chelating metal ions (Gardner, 1972). Citric acid is approved as a GRAS substance for miscellaneous and general-purpose usage when used in accordance with good manufacturing practices, in the acid form (21 CFR 184.1033) or as the calcium (21 CFR 184.1195), potassium (21 CFR 184.1625), or sodium salt (21 CFR 184.1751). The acceptable daily intake for humans is listed in Table 4.3.

FUMARIC ACID

Antimicrobial Properties

Fumaric acid (0.15%) served as an acidulant (Ough and Kunkee, 1974) and inhibitor of malolactic fermentation in wine (Pilone, 1975). Huhtanen (1983) and coworkers (1985) investigated the effect of various esters of fumaric acid on growth of *C. botulinum* types A and B in canned bacon. Monomethyl, dimethyl, and ethyl esters of fumaric acid in concentrations of 0.125%, 0.15%, or 0.2% showed considerable promise as a substitute for sodium nitrite in bacon. Cans packed with bacon and inoculated with *C. botulinum* swelled in 4 to 6 days at 30°C, whereas the presence of fumaric acid esters in the bacon prevented swelling for up to 8 weeks. Fumaric acid by itself was only slightly inhibitory. The n-monoalkyl fumarates and maleates esterified with C13–C18 alcohols also possessed significant antibotulinal activity (Dymicky et al., 1987). Methyl n-alkyl fumarates demonstrated lower activity, and di-n-alkyl fumarates were almost inactive (Dymicky et al., 1987). Medium chain-length alkyl esters had more activity than aliphatic acids (Dymicky and Trenchard, 1982).

The use of fumaric acid to effect a 5-log reduction of *E. coli* O157:H7 inoculated into unpreserved apple cider was investigated by Comes and Beelman (2002). The addition of 0.15% fumaric acid coupled with 0.05% sodium benzoate followed by holding at 25°C for 6 hours before refrigeration at 4°C for 24 hours achieved reduction. These same preservatives provided a similar reduction in less than 5 hours and 2 hours when held at 25°C and 35°C, respectively. In addition to reduction of *E. coli* O157:H7, total aerobic counts were reduced to levels below those achieved through pasteurization.

Fumaric acid was also more effective than lactic or acetic acid when used in meat products (Podolak et al., 1996a,b). Fumaric acid at concentrations of 1.0% and 1.5% applied for 5 seconds at 55°C to the surface of lean beef inoculated with *L. monocytogenes* and *E. coli* O157:H7 reduced levels by 1 and 1.3 logs, respectively. When used in vacuum-packaged ground beef patties, fumaric acid achieved the greatest reduction of aerobic, psychrotrophic, and coliform bacteria. It was speculated that the antimicrobial properties of fumaric acid were related to a lower pKa than lactic or acetic acids.

Monomethyl and monoethyl (0.2%) and dimethyl and diethyl fumarates (0.05%) or sodium propionate (0.1%) controlled fungal growth in tomato juice. The fumarate esters not only prevented mold growth on bread (Huhtanen et al., 1981; Islam, 1982), but the dimethyl and diethyl esters at a concentration of 20 mg per 2.6 L in the vapor phase were also effective as a mold preventive (Huhtanen and Guy, 1984).

Fumaric acid was more fungicidal against *Talaromyces flavus* ascospores than acetic, citric, malic, or tartaric acid based on weight when added to the heating medium. A 0.5% concentration of fumaric acid inactivated ascospores after 20 minutes of heating at 80°C, and lethality increased as the pH decreased from 5.0 to 2.5. However, malic, citric, and tartaric acids at a concentration of 2.5% (pH 2.5 to 5.0) were not lethal to *T. flavus* ascospores (Beuchat, 1988). Similar effects were noted with *N. fischeri* (Conner and Beuchat, 1987) heated in the presence of 2.0% acid concentration at 82°C. Fumaric, acetic, citric, and tartaric acids increased heat inactivation, but malic acid did not.

Toxicology

Weanling rats fed 0.1%, 0.5%, 0.8%, and 1.2% fumaric acid or rabbits fed sodium fumarate at a concentration of 6.9% (5% fumaric acid) showed no toxic effects (Packman et al., 1963). At the 1.5% level, rats showed an increased mortality rate and more atrophy of the liver (Fitzhugh and Nelson, 1947). The 2-year studies using rats fed 0.1% and 1.0% fumaric acid and 1.38% sodium fumarate or guinea pigs fed 1.0% fumaric acid did not show any abnormalities. Human feeding

trials using a 500 mg/day oral dose for 1 year were also negative. Because of these dosage levels, an LD₅₀ of 8,000 mg/kg was proposed (Levey et al., 1946).

Application and Regulatory Status

Fumaric acid is a nonhygroscopic, unsaturated, dicarboxylic acid having low water solubility. Although this additive has a strong acid taste, it blends with certain flavoring compounds to intensify the aftertaste of a flavor. Fumaric acid is used in fruit drinks, gelatin desserts, pie fillings, biscuit doughs, and wines. It increases the strength of gelatin gels. In addition to supplying acidity to a product, fumaric acid does display some antioxidant properties in fat-containing foods (Gardner, 1972). Fumaric acid and its salts are used for direct addition to food for human consumption (21 CFR 172.350). The acceptable daily intake for humans is listed in Table 4.3.

LACTIC ACID

Antimicrobial Properties

Lactic acid has been used more extensively for its sensory qualities than its antimicrobial properties in the past, although more recently this acid has been used as a rinse for beef, pork, and chicken carcasses. The inhibitory capacity of this acid lies in its reduction of pH to levels below which bacteria cannot initiate growth. In fermented foods, lactic acid coupled with other antigrowth factors excreted by lactic acid-producing microorganisms inhibits competing microorganisms.

Lactic acid was an excellent inhibitor of spore-forming bacteria at pH 5.0 but was ineffective against yeasts and molds (Woolford, 1975a). Lactic acid was about four times more effective than malic, citric, propionic, and acetic acid in limiting growth of *Bacillus coagulans*, the organism responsible for flat-sour spoilage in tomato juice (Rice and Pederson, 1954). Lactic acid was more inhibitory to *Mycobacterium tuberculosis* as the pH decreased (Dubos, 1950). Gill and Newton (1982) suggested that the inhibitory effect for psychrotrophic organisms was related to a decrease in pH rather than degree of dissociation. Cold-pack cheese normally prepared with lactic acid was reformulated with acetic acid and inoculated with *S. Typhimurium*. Destruction rates for *S. Typhimurium* were similar for both acids (Park et al., 1970).

Lactic acid sprays have been effective in limiting microbial growth on meat carcasses under a variety of storage conditions. A 1.0% to 1.25% concentration of acid sprayed on veal carcasses, followed by vacuum packaging, lowered microbial counts after storage for 14 days at 2°C, but a 2% concentration led to discoloration of the carcass surface (Smulders and Woolthuis, 1983; Woolthuis and Smulders, 1985). A 2% L-lactic acid treatment at pH 2.3 of veal tongues, combined with vacuum packaging and storage at 3°C, reduced aerobic mesophilic plate counts from 5.6 to 2.7 log CFU/cm² (Visser et al., 1988). Optimal decontamination was achieved when 1% lactic acid solution at 55°C was sprayed on beef carcasses immediately after dehiding and after evisceration (Prasai et al., 1991). Osthold et al. (1984) found that spraying beef and sheep carcasses with a combination of 1% lactic, 2% acetic, 0.25% citric, and 0.1% ascorbic acids selectively inhibited Enterobacteriaceae at 10°C.

Similar reductions in microbial counts occurred with a 1.25% lactic acid spray of beef carcasses followed by a treatment of hot-boned cuts with 2% acid, vacuum packaging, and storage for 10 days. Acid treatment when combined with vacuum packaging was more effective in prolonging shelf life than vacuum packaging alone (Smulders and Woolthuis, 1985). Enterobacteriaceae contaminated 50% of the untreated samples, which was reduced to 10% after treatment. A study using lactic acid sprays of skinned cow heads found that 1% acid was effective in significantly reducing total counts and extending shelf life by 3 days at 4°C and 1 day at 15°C and 20°C (Cudjoe, 1988). Because most other studies chose refrigeration storage temperatures, this study showed that lactic acid was effective at higher temperatures as well.

Hot-boned, sub-primal meat was injected with one of three treatments: 0.3 M calcium chloride solution (CAL, pH 5.1), 0.3 M lactic acid solution (LAC, pH 3.0), or a combination (1:1) CAL/LAC (COM, pH 2.5) at a volume equal to 10% of the initial sub-primal weight. Significantly higher aerobic plate counts were obtained from hot-boned cuts over 17 days postmortem compared to cold-boned cuts. Aerobic plate counts of meats injected with CAL were significantly higher than with LAC or COM. Furthermore, COM improved tenderness in muscle cuts, but control cuts retained more desirable flavor profile (Eilers et al., 1994).

Kotula and Thelappurate (1994) compared acetic acid and lactic acid solutions applied at two concentrations, 0.6% or 1.2%, and at two time intervals, 20 or 120 seconds, at 1°C to 2°C as a dip for rib eye steaks. Minimal reduction in total plate and *E. coli* counts was detected for beef dipped in acetic or lactic acid solutions compared with a water-dipped control at day 1. A residual effect was noted for the lactic acid-treated tissue in that microbial counts were significantly decreased compared to the control steaks after 9 days of storage, but this effect was not seen with acetic acid-treated tissue. Acid-treated samples were lighter in color as a result of leaching of the pigment during immersion, but shear values, moisture content, and sensory analysis were not affected by acid treatment.

Postchill beef rounds inoculated with *E. coli* O157:H7 or *S. Typhimurium* were subjected to an automated wash water system (Castillo et al., 1998; 2001a,b). The system alone produced a 3-log reduction. Sprays containing 4% lactic acid applied at 55°C for 30 seconds or at 65°C for 15 or 30 seconds led to unrecoverable levels of *E. coli*. When sequential treatments were employed using pre-chill and postchill lactic acid sprays, *E. coli* was reduced by 6.8 to 7.2 logs. Microbial counts obtained from ground beef made from the treated beef rounds that were subjected to the multiple sprays were significantly lower than samples receiving a postchill rinse, only suggesting that lactic acid can exert a continued antibacterial effect during the shelf life of the product.

Beef trim meat used to make ground beef is of particular concern because of its higher microbial load. Beef trim was fabricated to make predominantly beef trim lean (BTL) and beef trim fat (BTF) products that were inoculated with bovine fecal samples (Kang et al., 2001a,b). The inoculated meat was subjected to sprays of tap water (15°C to 17°C, 65 psi); 2% lactic acid (12°C to 15°C, 30 psi); hot water (30 psi) at 65°C, 71°C, 76°C, or 82°C; or hot air at 371°C, 426°C, 454°C, 482°C, or 510°C. In addition, combinations of the treatments were examined. All products were stored at 4°C and examined for aerobic, psychrotrophic, and presumptive lactic acid bacteria; total fecal coliforms; and *E. coli* biotype 1 counts. Greater reductions on all microbial loads were seen in BTF compared to BTL. Lactic acid application as the final step in a multiple-hurdle process led to a residual effect during storage for 7 days at 4°C. Similar work was conducted using lean pork trim (LPT) and fat-covered pork trim (FPT) (Castelo et al., 2001). As in beef trim, the FPT supported lower microbial numbers than LPT. Treatments containing lactic acid resulted in the lowest counts without affecting sensory qualities. Application of 95°C water alone or with 2% L-lactic acid was effective in reducing *E. coli* O157:H7 and *S. Typhimurium*-inoculated beef trimmings. Meat color was affected after application and through storage at 4°C, but odor was not affected (Ellebracht et al., 1999).

Acuff et al. (1987) compared a combination of 1% lactic, 2% acetic, 0.25% citric, and 0.1% ascorbic acids adjusted to pH 2.6 with either 1% lactic acid at pH 2.9 or 1% acetic acid at pH 3.3. There was little difference in bactericidal activity between the combination and either acid alone on sub-primal cuts of beef. Dixon et al. (1987) confirmed their findings using beef strip loins stored in polyvinyl chloride (PVC) film for 6 days or high-oxygen barrier film for 28 days. When temperature changed from 20°C to 70°C or lactic and acetic acid concentrations increased individually to 3%, aerobic bacterial counts and *S. Typhimurium* levels decreased approximately 1 log, <1 log for Enterobacteriaceae and 0.5 logs for *E. coli* (Anderson and Marshall, 1990a, b). The combination of acids performed as well as 1% lactic or acetic acids alone. Lactic acid was most effective against *S. Typhimurium* at 70°C, reducing numbers by 2 logs and Enterobacteriaceae by 1.5 logs (Anderson et al., 1992).

Lactic acid (2%), low-molecular-weight polylactic acid (2% PLA), and nisin (400 IU/ml) were used alone or in combination in beef to inhibit *L. monocytogenes* (Ariyapitipun et al., 2000). Beef was inoculated with *L. monocytogenes* before vacuum packaging and storage at 4°C. All treatments significantly reduced the number of *L. monocytogenes*; however, there was no significant difference between nisin, and low-molecular-weight polylactic acid, lactic acid, and polylactic acid. After 42 days of storage the initial inoculum of 5 logs was reduced to <1 log after using lactic acid, nisin, or nisin and lactic acid. Beef inoculated with *E. coli* O157:H7 was subjected to treatments of low-molecular-weight polylactic acid, lactic acid, and 200 IU/ml of nisin (Mustapha et al., 2002). After 28 days of storage, significant reductions were seen with all treatments, but no differences were seen between polylactic acid and lactic acid, and nisin did not enhance the effects of lactic acid against *E. coli* O157:H7. The use of these compounds was effective in reducing psychrotrophs and Enterobacteriaceae (Ariyapitipun et al., 1999). Beef cubes were inoculated with *L. monocytogenes* and treated with 2% lactic acid, 40,000 IU/ml nisin, and 3200 units/ml pediocin. These compounds reduced the level of *L. monocytogenes* by 1.7, 1.1, and 0.6 log/6 cm², respectively (El-Khateib et al., 1993).

A novel approach to shelf life extension was the use of lactic or acetic acid-impregnated calcium alginate gels applied to the surface of beef tissue (Siragusa and Dickson, 1992, 1993). Although the gels reduced populations of *L. monocytogenes* on beef tissue, noncoated tissue became dehydrated during the 7-day study. Lactic acid treatments alone were only 50% as effective as acid/alginate treatments, whereas acetic acid was more effective than the acetic acid/gel treatment. Numbers of *S. Typhimurium* were reduced more by exposure to acetic acid/gel treatment than acetic acid alone.

Lactic acid (2%), acetic acid (0.5%), or sodium lactate (4%) alone or in combination with pulse electric field or freezing was ineffective in reducing numbers of *E. coli* O157:H7 inoculated into beef trimmings (Bolton et al., 2002). Therefore, these treatments were not deemed usable in the control of *E. coli* O157:H7 in beef burgers.

The combination of acids and heat treatment led to reduction of spore-forming organisms in frankfurters. Frankfurter emulsions incorporated with acids to adjust pH to 5.2 or 4.6 for *Bacillus stearothermophilus* and pH 4.5 and 4.2 for *B. coagulans* were heated to 121°C and 105°C or 110°C. Greater inactivation was noted for both organisms at the lower pH level when acetic or lactic acids were used but not with citric, malic, or hydrochloric acid (Lynch and Potter, 1988).

The use of lactic acid for reduction of microbial numbers or pathogens has been similarly applied to pork products. Woolthuis et al. (1984) effectively reduced total plate counts, Enterobacteriaceae, and Lactobacillaceae counts by 2 to 3 logs, by immersing porcine livers in 0.2% lactic acid for 5 minutes, vacuum packaging, and storing for 5 days at 3°C. A 1% concentration of lactic acid (pH 2.8, 55°C) had little effect on the aerobic plate counts taken from the surface of pork carcasses. *Salmonella* species or *Listeria* species were not recovered nor were sensory characteristics affected (Prasai et al., 1992). By increasing the concentration of lactic acid to 2%, numbers of *Salmonella* species and *Campylobacter* species were reduced immediately and remained lower 24 hours after slaughter (Epling et al., 1993). Van Netten and Hust In't Veld (1994) developed a pork skin model system to determine the effect of lactic acid decontamination on microbial counts and changes in predominance of the microflora. Five treatments were delivered to the pork skins: water-treated (control) or exposure at 21°C for 120, 180, 240, or 360 seconds to 2% lactic acid. Not only did the microbial counts decrease as the time of decontamination increased, but the population of microorganisms shifted. Lactic acid was effective in decreasing the number of Enterobacteriaceae as well as other Gram-negative mesophilic and psychrotrophic spoilage organisms. Elimination of these groups shifted the population to Gram-positive bacteria and yeasts. Psychrotrophic, Gram-negative bacteria were the most sensitive to lactic acid, followed by mesophilic Enterobacteriaceae, psychrotrophic Gram-positive bacteria; lactobacilli and yeasts were the least sensitive.

The effects of 3% lactic acid at 55°C were studied using pork fat and lean tissue artificially inoculated with 10⁴ to 10⁵ CFU/cm² of *L. monocytogenes*, *Y. enterocolitica*, *Aeromonas hydrophila*,

P. fragi, or *Brochothrix thermosphacta* (Greer and Dilts, 1995). After inoculation, tissue was dipped for 15 seconds in water or lactic acid and held for 15 days at 4°C. The spoilage organisms, *P. fragi* and *B. thermosphacta*, grew on both tissue types after acid treatment, although tissue treated with acid affected the growth rate. None of the pathogens grew on lean tissue, and *A. hydrophila* numbers declined by 5 logs within 11 days of storage. Acid treatments of fat tissues led to the reduction of pathogen numbers to nondetectable numbers within 4 days.

Lactic acid dips have also been used successfully in the poultry industry. Total microbial numbers from skin of birds immersed for 15 seconds at 19°C in 1% or 2% lactic acid at pH 2.2 decreased from 5.2 to 3.7 log CFU/g (van der Marel et al., 1988). Levels of psychrotrophs decreased from 3.9 to 2.7 log CFU/g and Enterobacteriaceae from 3.3 to 2.6 log CFU/g. Higher concentrations of the acid did not ensure greater decontamination nor did repeated treatments. A 10% lactic acid and sodium lactate-buffered acid spray (pH 3.0) for chicken legs increased the shelf life from 6 to 12 days at 6°C, and a 2% lactic acid dip at pH 2.3 prolonged shelf life to 8 days (Zeitoun and Debevere, 1990). These treatments inhibited hydrogen sulfide-producing bacteria, such as *Pseudomonas* species, that contributed to spoilage. The treatments did not affect sensory quality.

Lactic acid (1%) added to both chill water (0°C to 1.1°C, pH 2.8) and scald water (54°C/2 minutes) reduced the bacterial level of broilers inoculated with *S. Typhimurium* to almost nondetectable numbers. Lactic acid added to scald water alone had minimal effect on reducing the numbers of contaminated birds. The number of *Salmonella*-positive birds was also reduced as a function of time of the dip (Izat et al., 1990a). Lactic acid added to broiler chill water resulted in the development of a brown coloration. In an effort to eliminate carcass discoloration, reduced levels of lactic acid (0.25%, pH 2.88 or 0.5%, pH 2.62) were combined with propylene glycol (20%) in chill water. *Salmonellae* were eliminated from broiler carcasses after a 1-hour exposure; however, discoloration still occurred and propylene glycol contributed an objectionable flavor (Izat et al., 1990b). Chicken skin subjected to washing treatments using 1% lactic acid led to significant reductions of *Salmonella* species and *L. monocytogenes* compared with monosodium phosphate, sodium tripolyphosphate, sodium acid pyrophosphate, or sodium hexametaphosphate. Consumer evaluation of chicken treated with a 0.5% lactic acid/0.5% sodium benzoate dip concluded that sensory qualities were acceptable (Hathcox et al., 1995; Hwang, and Beuchat, 1995). Pretreatment of broiler carcasses with lactic acid buffer increased shelf life by 6 to 7 days at 4°C and 5 to 6 days at 7°C. When treated broilers were packaged using modified atmospheres, shelf life was lengthened to >36 and 35 days at 4°C and 7°C, respectively (Sawaya et al., 1995).

Lactic acid was more effective than acetic acid when used as a 10-minute rinse for fresh-cut vegetables. Although *L. monocytogenes* counts were reduced by 0.5 and 0.2 logs, respectively, other compounds, such as chlorine or chlorine dioxide, produced similar reductions of approximately 1 log (Zhang and Farber, 1996).

The interaction of sodium chloride (0.5% to 4%) and pH (4.2) was examined for *E. coli* O157:H7 grown in TSB at 37°C (Casey and Condon, 2002). *E. coli* O157:H7 rapidly died at pH 4.2 in media containing lactic, acetic, or formic acids. *E. coli* demonstrated a 10-fold decrease in numbers when grown in media containing sodium chloride, acidified with lactic acid; however, in sodium chloride-free, acidified media, *E. coli* decreased 10,000-fold. Sodium chloride offered a degree of protection against the inhibitory activity of lactic acid when the culture was incubated in the medium containing both ingredients, but no protective effect was observed if the sodium chloride was added to the medium 45 minutes after the inoculation. This protective effect was even more pronounced with acetic acid (100,000-fold). The sodium chloride-sparing effect was partially attributed to a reduction in water activity causing the cell size to shrink as a result of loss of water in the cytoplasm and a concomitant increase of internal pH from 5.23 in cells exposed to acid to 5.79 in acid-free media.

S. aureus grown at 37°C or 46°C in the presence of 1 M sodium chloride had marked differences in its sensitivity to acetic and lactic acids (El-Banna and Hurst, 1983). Cells grown at 46°C were more heat resistant and better able to grow in media adjusted with acetic and lactic acids to pH 5.0 than cells grown at 37°C.

El-Gazzar et al. (1987) found that *A. parasiticus* NRRL 2999 grown in a laboratory medium containing 0.5% and 0.75% lactic acid adjusted to pH levels of 3.5 or 4.5 using HCl or NaOH and incubated for 10 days at 28°C produced more aflatoxin B₁ than other cultures at 3 days of incubation. With increasing levels of lactic acid, cultures decreased production of aflatoxin G₁. Luchese and Harrigan (1990) also noted an increase in aflatoxin production adjusted to pH 4.2 using HCl or lactic acid stimulated by substituting half of the carbon content by lactate and a reduction in production at pH 6.8. Aflatoxin production also increased during growth of *A. parasiticus* in association with *L. lactis*. A predictive model for growth of *E. coli* in response to temperature, water activity, pH, and lactic acid concentration has been developed (Mellefont et al., 2003; Presser et al., 1998; Ross et al., 2003).

Toxicology

The LD₅₀ of lactic acid for test animals varied according to animal species (Table 4.2). Infants died after consuming milk acidified with an unknown quantity of lactic acid. The autopsy revealed hemorrhaging and gangrenous gastritis (Young and Smith, 1944). In two other instances, infants suffered stricture of the esophagus following ingestion of milk acidified with 1 teaspoon of 85% (Pitkin, 1935) or 87.5% lactic acid (Trainer et al., 1945). Premature infants fed acidified milk containing the isomeric forms of lactic acid, D (-) or DL, developed acidosis, weight loss, dehydration, and vomiting. Therefore, Ballabriga et al. (1970) recommended that the L (+) form should be used in feeding premature infants. The Food and Agriculture Organization of the United Nations (Food and Agriculture Organization, 1973) supported this view. Hamsters fed a cariogenic diet incorporating lactic acid in the drinking water (40 mg/100 ml) or in the feed (45.6 mg/100 g) did not differ in growth rate compared with control groups (Granados et al., 1949). Some enamel decalcification was apparent in the experimental groups.

Application and Regulatory Status

Lactic acid is a hygroscopic, syrupy liquid having a moderately strong acid taste. Lactic acid is used in the manufacture of jams, jellies, sherbets, confectionery products, and beverages. It is used to adjust acidity in brines for pickles and olives. Calcium lactate is used as a firming agent for apple slices, to prevent discoloration in fruit, and in baking powders. Lactic acid is approved as a GRAS substance for miscellaneous or general-purpose usage (21 CFR 184.1061). The acceptable daily intake for humans is listed in Table 4.3.

LACTATES

Antimicrobial Properties

The salt form of lactic acid has been used successfully as an antimicrobial agent because it provides a slight salty taste that enhances meat flavor, retains color, contributes to water-holding capacity, improves juiciness, increases meat yields, appears naturally in meat products, and extends the shelf life of products. For an excellent review of the antimicrobial nature of sodium lactate, see Shelef (1994) and De Wit and Rombouts (1990).

The MIC of sodium lactate was investigated under optimum growth conditions (pH 6.5, 20°C) for a number of bacteria and yeasts isolated from spoiled and unspoiled meat products (Houtsma et al., 1993). MIC values for *Salmonella* species ranged from 714 to 982 mM and a narrower range for *L. monocytogenes* and *L. innocua* from 804 to 982 mM. Gram-negative bacteria including *Pseudomonas* and *Yersinia* had a comparable range to *Salmonella*, although *Campylobacter* was particularly sensitive with an MIC of 179 mM. Gram-positive bacteria including lactic acid bacteria, *Carnobacterium*, *Lactococcus*, *Brochothrix*, *Bacillus*, and *Staphylococcus* ranged from 268 for *S. aureus* and *Lactobacillus coryniformis* to 804 mM for *Brochothrix*. Yeasts were very broad in

their sensitivity, with *Debaryomyces* and *Candida* being the most resistant at 1161 and 1339 mM, respectively, which might explain their prevalence in spoiled meat products.

The effect of sodium lactate on toxin production, spore germination, and heat resistance was tested on proteolytic *C. botulinum* strains in peptone yeast extract medium (Houtsma et al., 1994a). Toxin production occurred within 14 days of incubation at 15°C for cultures grown in 0% and 1% sodium lactate but was delayed in the presence of 2% sodium lactate until day 21; no toxin formation was detected after 49 days of incubation in media containing 3% sodium lactate. At a higher temperature of 20°C, toxin formation was noted within 5 days for 0%, 11 days for 1.5%, and 15 days for 2.5%; no growth or toxin formation was detected after 32 days of incubation for media containing 4.0% sodium lactate. At 30°C, toxin production was detected in media containing 4% sodium lactate within 11 days; however, spore germination did not lead to growth after 7 days. Sodium chloride at the same water activity as sodium lactate (0.982) did not inhibit toxin production, indicating that inhibition was not the result of a lowering of water activity.

Lactic acid/sodium lactate and acetic acid/SA were studied in a BHI broth at pH 7, 6, 5, and 4 using 0.1, 0.5, 1.0, and 2 M concentrations (Buchanan et al., 1993). Survivor curves were developed using *L. monocytogenes* as the target organisms. As expected, the rate of inactivation was dependent on the acid, its concentration, and the resultant pH. Lactic acid was more effective at higher pH levels whereas acetic acid was more effective at lower pH levels and there was a correlation with the concentration of dissociated acid.

The effect of sodium lactate and sodium chloride on the growth of *L. innocua* was modeled in broth adjusted to pH 5.5, 6.0, 6.5, and 7.0 at 4°C, 10°C, 20°C, and 30°C (Houtsma et al., 1994b). The pH level was influential in inhibition by sodium lactate but not by sodium chloride. As expected, inhibition by sodium lactate was directly related to pH and temperature, with lower amounts of sodium lactate needed as pH and temperature dropped. This model was then evaluated for use in a bologna-type sausage system (Houtsma et al., 1996).

Chen and Shelef (1992) suggested that in addition to pH change, lactates also contributed to lowering the a_w of a food. *L. monocytogenes* was studied in a cooked meat model system containing varying moisture levels and 4% lactate. This level was listeristatic at a moisture of >55%, but the combination of 2% or 3% lactate and 2% sodium chloride at the same moisture level was inhibitory. Sodium, calcium, and potassium lactate were equally effective.

Salts of lactic acid are equally as effective in muscle food systems. Pork liver sausage was formulated with sodium, potassium, or calcium lactate at concentrations of 2%, 3%, or 4% and inoculated with 10^4 to 10^5 *L. monocytogenes* per gram of sausage. Samples were stored up to 50 days at 5°C and up to 10 days at 20°C. Numbers increased to $>10^8$ CFU/g after 50 days at 5°C in sausages not containing lactates but were reduced with 2% or 3% sodium lactate and 2% potassium lactate. Calcium lactate (2%) reduced populations below the inoculum level. At the abuse temperature of 20°C, 4% sodium or potassium lactate achieved reductions in *L. monocytogenes*, comparable to a 3% concentration of calcium lactate. Addition of 2% sodium chloride enhanced the listericidal activity of the lactates (Weaver and Shelef, 1993). A subsequent study (Shelef and Potluri, 1995) examined the effect of 3% sodium or calcium lactate added to pork liver sausage and various heat treatments. As expected *L. monocytogenes* was inhibited by combination of 3% lactate and heat sterilization (121°C for 15 minutes) followed by a water bath heat process (70°C internal temperature) and least affected when lactate was added to sausage without heat treatment. The calcium form was found to be more inhibitory than the sodium form. Calcium lactate was also more effective than sodium lactate in retarding growth of *E. coli* O157:H7 and *S. Typhimurium* when stored for 4 days at 20°C.

Fresh pork sausage formulated with 0% to 3% sodium lactate was vacuum packaged and stored for up to 28 days at 4°C (Brewer et al., 1991, 1995). Using 10^8 CFU/g as a microbial end point, 1% sodium lactate delayed growth for 10 days, whereas a 2% level extended the time to 24 days. Potassium lactate (4%) and sodium chloride (3%) reduced growth somewhat at 5°C. There did not appear to be a synergistic effect between a 4% concentration of lactate combined with 140 ppm

potassium nitrite. Red color was preserved by using 2% to 3% sodium lactate. Pork sausages manufactured with 25% fat or 8% fat with and without 3.0% potassium lactate showed differences in spoilage rates. Lactates inhibited the growth of psychrotrophic organisms and delayed the pH decline (Bradford et al., 1993).

Cured meats, such as wieners, rely on sodium nitrite to inhibit growth of *C. botulinum*. Over the storage life of the product, nitrite levels are reduced. Retention of nitrite was linked to higher pH levels and was unaffected by the incorporation of 1.2%, 2.4%, or 3.6% sodium lactate into the product (Kilic et al., 2002). Frankfurters containing 2.0% or 3.0% potassium lactate were inoculated with a five-strain cocktail of *L. monocytogenes* and stored at 4°C and 10°C for up to 90 and 60 days, respectively (Porto et al., 2002). Both concentrations of potassium lactate significantly inhibited or delayed the growth of *L. monocytogenes* during storage at refrigerator and abuse temperatures. Sodium lactate (2%) was also effective in extending the shelf life of lower-fat frankfurters to 6 weeks compared to 3 and 4 weeks for low-fat and high-fat control frankfurters (Bloukas et al., 1997). Another study (Bedie et al., 2001) compared the use of 3% or 6% sodium lactate, 0.25% or 0.5% SA or sodium diacetate as an antilisterial process for frankfurters. Sodium lactate at a 6% concentration or 0.25% sodium diacetate were the most effective treatments throughout the 120-day shelf life conducted at 4°C with minimal change in pH. The growth of *L. monocytogenes* was delayed for 4 and 12 weeks at 7°C and 3°C in unsmoked, uncured bratwurst manufactured with 3.4% sodium lactate/0.1% sodium diacetate (Glass et al., 2002). Levels of >3% sodium lactate or 1% each of lactate and diacetate were needed to prevent growth of *Listeriae* for 60 days at 4.5°C

Lactate influenced the growth of lactic acid bacteria in meat based on the pH level and the atmospheric conditions under which postrigor meat was held (Grau, 1980). At pH 6.1, the amount of L-lactate ordinarily found in muscle was not inhibitory to any of the strains. A 100-mM concentration of sodium lactate buffered to pH 5.5 in a meat system prevented anaerobic growth of *Serratia liquefaciens*, *Y. enterocolitica*, *Enterobacter cloacae*, and *A. hydrophila* and aerobic growth of the aeromonads (Grau, 1981). When raised to 210 mM, sodium lactate permitted aerobic growth of *B. thermosphacta* down to pH 5.5. A 3% concentration of sodium lactate at pH 7.05 and 6.5 reduced surface discoloration in chubs of fresh sausage and extended shelf life from 10 to 20 days. In retail products, sodium lactate caused more rapid surface discoloration (Lamkey et al., 1991). Sodium lactate was less effective against microorganisms when sausage contained soy concentrate.

Papadopoulos et al. (1991a,b,c) injected beef top rounds with 0% to 4% sodium lactate, then cooked and vacuum-packaged the roasts. Although the aerobic plate counts for control meat increased from 2.7 to 8.0 log CFU/cm² after 84 days of storage, the plate counts of treated roasts increased to 6.5 log CFU/cm² in the same period. The microflora changed from predominantly staphylococci and micrococci at 0 days to coryneform, micrococci, lactobacilli, and yeasts at 42 days to homofermentative and heterofermentative lactobacilli and streptococci at 84 days. Lactate increased cooking yields, enhanced flavor at the 1% level by adding a salty taste, but produced a mild throat irritation at a 4% concentration. Although Shelef and Yang (1991) suggested a possible a_w effect for sodium lactate, Papadopoulos et al. (1991b) did not find this effect in their studies.

Minced beef containing 0%, 2.4%, or 4.8% sodium lactate was inoculated with *Y. enterocolitica* or *L. monocytogenes* and heated to 55°C to simulate a sous vide process (McMahon et al., 1999). The pH of the product was unaffected by incorporation of sodium lactate. The heat resistance of the two organisms was significantly lowered in beef containing either of the two concentrations of sodium lactate.

Cooked beef top rounds containing 1%, 2%, 3%, or 4% sodium lactate were inoculated with *L. monocytogenes*, *S. aureus*, *S. Typhimurium*, *C. perfringens*, or *E. coli* O157:H7 and stored at 10°C. Sodium lactate at concentrations of 3% and 4% were generally effective in limiting growth of *L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157:H7 (Miller and Acuff, 1994).

Shelef and Yang (1991) found that a 5.0% concentration of sodium or potassium lactate delayed the growth of *L. monocytogenes* in TSB and 4% limited growth of *L. monocytogenes* in comminuted chicken or beef when held at 35°C, 20°C, or 5°C. They concluded that the two salts gave comparable results and could be used interchangeably, suggesting that the lactate moiety was the effective component. Lactate contributed to water-holding capacity and increased cooking yields.

Maas et al. (1989) demonstrated that sodium lactate was effective in retarding toxin formation by *C. botulinum* in comminuted raw turkey using levels of 2%, 2.5%, 3%, or 3.5%. The meat was cooked to an internal temperature of 71.1°C, vacuum packaged, and stored at 27°C. Toxicity occurred within 3 days in turkey without lactate and within 4 to 5, 4 to 6, 7, or 7 to 8 days for each concentration, respectively. Organoleptically, the 2.5% concentration was acceptable, whereas off-flavors developed in meat containing the 3.5% concentration. They also noted that the inhibitory component was the lactate moiety, not sodium. Maas et al. (1989) suggested that a possible mechanism for the action of lactate was the inhibition of a major anaerobic energy metabolism pathway necessary for growth. Unda et al. (1991) also found that 2% lactate inhibited the growth of *C. sporogenes*.

The influence of 2%, 5%, or 10% lactic acid/sodium lactate buffer rinses (pH 3.0) alone or combined with modified atmosphere packaging was examined on the survival of *L. monocytogenes* and the shelf life of chicken legs (Zeitoun and Debevere, 1991). Some treated chicken was packaged without further modification, but other samples were placed into packages that were gas flushed with a 90% carbon dioxide/10% oxygen mixture; all were stored at 6°C. Excised skin tissue was analyzed for *Listeriae* for up to 17 days posttreatment. Product was considered spoiled when microbial numbers reached 7 to 8 log CFU/g. Odor concomitant with growth of psychrotrophic spoilage organisms occurred in untreated samples within 8 days and within 9, 10, and 13 days for the 2%, 5%, and 10% acid spray, respectively. Modified atmosphere packaging (MAP) extended storage life for products without acid sprays to 17 days and even longer with sprays. Reduction in production of hydrogen sulfide and other sulfur-containing compounds resulted from the decreased populations of psychrotrophs. Levels of *L. monocytogenes* increased by only 0.56 log during 17 days of storage for the legs treated with 10% buffer combined with MAP. Sodium lactate (3.3%) was very effective in reducing numbers of *B. thermosphacta* and *E. coli* in a chicken model system after 14 days at 22°C but only delayed the growth of *Lactobacillus alimentarius* by about 2 days (Lemay et al., 2002).

Lactates have been combined with other compounds in multiple-barrier studies. Vacuum-packaged beef was treated with 2% sodium lactate, pediocin (1400 U/g), nisin (1400 U/g, Nisaplin (500 IU/g), or Microgard™ (2%) (Rozbeh et al., 1993). Of all the treatments, sodium lactate was the most effective during an 8-week storage period at 3°C. Lactic acid at 25 to 30 mEq in combination with sodium nitrite, potassium sorbate, or glycerol monolaurate enhanced inhibition of *S. aureus* grown anaerobically (Smith and Palumbo, 1980). Sodium lactate at the same concentration had no effect. Notermans and Dufrenne (1981), however, found that glyceryl monolaurate at a concentration of 5 g/kg of a meat slurry inhibited *C. botulinum* types A, B, and E only when lactic acid was used to reduce pH below 5.2. Beef roasts containing sodium lactate, glycerol monolaurin, or sodium gluconate and inoculated with *L. monocytogenes* and *C. sporogenes* were stored at 2°C, 7°C, 10°C, and 25°C. Sodium lactate was more effective at reducing pathogen numbers at levels up to 3.5% than sodium gluconate, which did not provide significant control (Stillmunkes et al., 1993).

Harmayani et al. (1991) looked at the interactive effects of sodium alginate and calcium or sodium lactate used in restructured meats on *P. fragi* and *S. Typhimurium*. Ground beef containing sodium alginate at concentrations up to 0.8%, calcium lactate up to 0.3%, or a combination of the two was inoculated with the two organisms and held at 5°C for 5 days. None of the treatments affected the growth of either organism. A 3% concentration of sodium lactate only minimally affected the growth of *S. Typhimurium* but did inhibit *P. fragi* at even at the 4% level.

Raw and cooked ground beef were prepared with 1.8% sodium lactate, 0.1% sodium erythorbate, 1% kappa carrageenan, and an alginate binder. The products were inoculated with *L. monocytogenes* and stored aerobically at 4°C. In general, sodium lactate was more effective than the other additives in inhibiting growth of *L. monocytogenes* and total aerobic plate counts (Harmayani et al., 1993). Similar studies used low-fat ground beef patties formulated with carrageenan, encapsulated salt, and hydrolyzed vegetable protein and containing 1%, 2%, or 3% potassium lactate. Carrageenan had no effect on bacterial loads, but the addition of 2% or 3% potassium lactate reduced microbial numbers. Some discoloration and lipid oxidation occurred, thus affecting the sensory qualities of the meat (Egbert et al., 1992).

Comminuted salmon was mixed with sodium lactate, sodium chloride, and sodium nitrite; inoculated with *L. monocytogenes*; vacuum packaged; and stored at 5°C or 10°C. Sodium lactate displayed synergistic activity with nitrite and increasing concentrations of sodium chloride; a 2% lactate and 3% sodium chloride inhibited *L. monocytogenes* for up to 50 days at 5°C. A 3% concentration of lactate in combination with 3% sodium chloride or with 125 ppm sodium nitrite and 3% sodium chloride was effective at 10°C (Pelroy et al., 1994).

Application and Regulatory Status

Calcium (21 CFR 184.1207), potassium (21 CFR 184.1639), and sodium lactates (21 CFR 184.1768) are also approved. The acceptable daily intake for humans is listed in Table 4.3.

MALIC ACID

Antimicrobial Properties

Malic acid is inhibitory to fungi and bacteria, probably as a direct effect of pH manipulation. Nunheimer and Fabian (1940) found that malic acid was inhibitory against *S. aureus* at pH 3.98.

Toxicology

Malic acid fed to rats at levels of 500 and 5000 ppm in the basal diet had no effect; at 50,000 ppm, however, food consumption decreased and growth rate declined (Hazelton Laboratories, 1971a). Dogs fed the same three levels of malic acid showed no effects using the same parameters (Hazelton Laboratories, 1971b). Reproductive experiments using rats fed 1000 and 10,000 ppm malic acid before mating showed no significant differences from the controls (Hazelton Laboratories, 1970).

Application and Regulatory Status

Malic acid is a nonhygroscopic, dicarboxylic acid with high water solubility. It has a strong acid flavor but does not have the same buildup of acid taste as other acids. It is used in sherbets and ices, fruit preserves, jams, jellies, and beverages, primarily for its flavoring and acidification characteristics (Gardner, 1972). Malic acid is approved as a GRAS substance for miscellaneous and general-purpose usage (21 CFR 184.1069). The acceptable daily intake for humans is listed in Table 4.3.

PROPIONIC ACID

Antimicrobial Properties

Propionic acid inhibited spore-forming bacteria, especially rope bacteria (*B. subtilis*), at pH 6.0. As the pH decreased to 5.0 or 4.0, the acid inhibited yeasts and molds but not to the same extent as bacteria (Woolford, 1975b). Chung and Goepfert (1970) compared various organic acids to determine the highest pH level that inhibited growth of *S. anatum*, *Salmonella* Senftenberg, and

Salmonella Tennessee when all other parameters were at their optimum. Propionic acid at pH 5.5 inhibited the growth of *Salmonellae* at a higher pH level than other acids (pH 5.4, acetic; pH 5.1, adipic; pH 4.6, succinic; pH 4.4, lactic; pH 4.3, fumaric and malic; pH 4.1, tartaric; pH 4.05, citric). Cherrington et al. (1991) determined that 0.5 to 0.7 M propionic acid at pH 5.0 was more inhibitory in 60 minutes compared to formic acid in 3 hours for *E. coli* K12 and *Salmonella*. At a 5-mM concentration, the rate of RNA (ribonucleic acid), DNA (deoxyribonucleic acid), protein, lipid, and cell-wall synthesis decreased, which led to an increase in cell mass without cell division and a decrease in viability (Cherrington et al., 1990). Eklund (1985) concluded that propionic acid was not as inhibitory as benzoic acid for *P. aeruginosa*. Much higher concentrations of propionic acid were bacteriostatic to *B. subtilis*, *E. coli*, *S. aureus*, and *Candida albicans*.

The salts of propionic acid are also effective antimicrobial agents. Calcium propionate prevented rope formation caused by *B. mesentericus* (*subtilis*) in bread dough at a level of 0.188%, pH 5.8, or at a level of 0.156%, pH 5.6 (O'Leary and Kralovec, 1941). Incorporation of calcium propionate into coatings for ready-to-eat products was effective in reducing problems with *L. monocytogenes* (Janes et al., 2002). Zein (Z) film coatings were dissolved in propylene glycol (ZP) or ethanol (ZE) with and without nisin (N; 1,000 IU/g) or calcium propionate (CP; 1%) and coated onto ready-to-eat chicken samples dipped in *L. monocytogenes*. Products were stored for up to 24 days at 4°C or 8°C. Nondetectable levels of the pathogen were found in products ZEN, ZPNCP, or ZENCP.

Sodium propionate at levels of 0.1% to 5% delayed growth of *S. aureus*, *Sarcina lutea*, *Proteus vulgaris*, *L. plantarum*, *Torula* species, and *S. ellipsoideus* by as much as 5 days. This substance not only postponed spoilage in fresh figs, syrup, applesauce, cherries, and berries but also prevented spoilage in vegetables having a more neutral pH, such as lima beans and peas (Wolford and Anderson, 1945).

Turkey breast meat was formulated with sodium salts of lactate, acetate, pyruvate, citrate, and propionate to a target level of pH 6.0, inoculated with *C. botulinum*, and held at 28°C for 0 to 18 days. At periodic intervals, samples were removed from storage and tested for production of neurotoxin. Toxin production occurred after 2 days for pyruvate, 3 for citrate, 4 for lactate and acetate, and 5 with a 2% concentration. At a 6% concentration, toxicity occurred after 7 days for pyruvate; 18 days for citrate; and greater than 18 days for propionate, acetate, and lactate. Citrate was most effective based on molarity (Miller et al., 1993).

In laboratory studies examining the effect of sodium propionate on *L. monocytogenes*, the organism was able to grow at up to a 0.3% concentration in tryptose broth adjusted to pH 5.6 and incubated at 4°C, 13°C, 21°C, and 35°C. Reduction of pH to 5.0 minimized growth at 13°C, 21°C, and 35°C and prevented growth at 4°C (El-Shenawy and Marth, 1989a). When media containing 0.3% sodium propionate was adjusted to pH 5.6 with acetic, tartaric, citric, or lactic acids in media (El-Shenawy and Marth, 1992), the lag phase of *L. monocytogenes* grown at 13°C was extended by 7, 7, 5, and 4 days, respectively. At pH 5.0 and 13°C, the levels of *L. monocytogenes* were undetectable. Sodium propionate at a 0.3% concentration was also effective at pH 5.0 in reducing numbers of *L. monocytogenes* when used in conjunction with acetic acid in cold-pack cheese food (Ryser and Marth, 1988). Exposure of *L. monocytogenes* to 8% sodium propionate for 60 minutes caused injury (Buazzi and Marth, 1992).

Emphasizing the multiple-barrier concept in retarding growth, Golden et al. (1995) examined the combination of sodium propionate and SA at a number of concentrations, pH levels, and temperatures. A three-strain mixture of *L. monocytogenes* was inoculated into BHI broth containing 0.08% EDTA, 0.02% ascorbic acid, and 0.9% sodium propionate or SA; adjusted to pH 4.5, 4.0, 3.5, or 3.0 with hydrochloric acid; and stored at 28°C, 19°C, or 4°C. Inactivation of *L. monocytogenes* was the result of incubation temperature, concentration of the organic acid, degree of dissociation of the acid, and pH. Propionic acid was more effective than acetic acid as an antimicrobial, particularly at the higher pH level. The acids contributed to approximately 85% of the antimicrobial activity with 14% contributed by ascorbic acid; little activity was ascribed to EDTA in combination with the acids.

The effect of fat concentration on the efficacy of antimicrobial agents against *L. monocytogenes* was studied in pork liver beaker sausage (Hu and Shelef, 1996). Sausage batter was mixed with 0.2% sodium propionate, 1.8% sodium lactate, and 0.1% sorbic acid or potassium sorbate. Increasing the fat content from 22% to 67% decreased the growth of *L. monocytogenes* by only 1.5 logs. The antimicrobial activity of sodium propionate was most affected at 4°C by fat concentration with a 1.5 log reduction of *L. monocytogenes* at 22% fat compared to 2.8 log reduction at 67% fat; with sodium lactate, 0.5 and 1.8 log reductions were observed, respectively.

Small amounts of β -alanine could overcome bacteriostatic action of sodium propionate for *E. coli*. However, β -alanine could not reverse the inhibitory effect of propionic acid in *Aspergillus clavatus*, *B. subtilis*, *Pseudomonas* species, or *Trichophyton mentagrophytes*. The inhibitory action could be the result of an interference with β -alanine synthesis (Wright and Skeggs, 1946).

Propionic acids and their salts are primarily inhibitory to molds; however, some species of *Penicillium* grew on media containing 5% propionic acid (Heseltine, 1952b). Propionic acid at a concentration of 0.1%, pH 4.5, reduced growth and aflatoxin formation of *A. flavus*; at 0.2%, no growth occurred. This inhibitory effect was more pronounced with the addition of acid at the time of inoculation, rather than later (Ghosh and Häggblom, 1985).

Propionic acid at a concentration of 2435 ppm was more effective than acetic acid in limiting growth of *Fusarium oxysporum* but less effective than sorbic acid and potassium sorbate. Spore germination was inhibited by 1402 ppm (Tzatzarakis et al., 2000).

Concentrations of propionic acid and propionates ranging from 8% to 12% were effective in controlling mold growth on the surface of cheese and butter (Deane and Downs, 1951; Ingle, 1940). A 5% calcium propionate solution acidified to pH 5.5 with lactic acid was as effective as a 10% unacidified solution in preventing surface mold growth on butter (Olson and Macy, 1945). Molds were inhibited by less calcium propionate by weight than sodium propionate. Not only was the final pH of the substrate critical, but also various organisms displayed different tolerances to the compounds (Olson and Macy, 1940).

A novel use of propionic acid to retard spoilage of bread was investigated by Gardner et al. (2001). Adding yeast extracts previously fermented by *Propionibacterium freudenreichii* provided a source of propionic acid in bread formulation. Bread produced with the extracts contained less ethanol and supported less spoilage by molds.

Mold spoilage is a significant economic problem in bakery products. Although mold spores are destroyed during baking, postprocess contamination from the atmosphere, cooling surfaces, and wrapping materials reintroduce molds. *Eurotium amstelodami*, *herbariorum*, *rubrum*, and *repens* were inoculated by needle into cake analogues prepared with calcium propionate, sodium benzoate, and potassium sorbate at a level of 0.3% by weight. All preservatives were effective at pH 6.0 and a water activity of 0.8 to 0.85 (Guynot et al., 2002). Subsequent studies (Marin et al., 2002a,b) examined several levels of calcium propionate, potassium sorbate, and sodium benzoate (0.003%, 0.03%, and 0.3%) at pH 4.5, 6.0, and 7.5 and water activity of 0.8, 0.85, and 0.93 in a model agar system on the retardation of growth of *E. amstelodami*, *herbariorum*, and *rubrum*, *A. flavus* and *niger*, and *Penicillium corylophilum*. None of the preservatives were effective at neutral pH. Suboptimal doses (0.03%) led to enhanced growth of *Aspergillus* and *Penicillium* isolates.

Toxicology

Orö and Wretland (1961) determined the LD₅₀ of propionic acid for mice, and Hara et al. (1963) determined the LD₅₀ of calcium and sodium propionate for rats (Table 4.2). Studies involving albino rats fed propionic acid at 50 cm³/kg of rice for 110 days showed umbilicate or warty lesions on the stomach (Mori, 1953). Additional studies using calcium and sodium propionate fed to mice, rats, and humans showed no toxic effects (Graham et al., 1954; Graham and Grice, 1955; Hara, 1965; Harshbarger, 1942). There is evidence that the sodium salt has some local antihistaminic activity (Heseltine, 1952a).

Application and Regulatory Status

Propionic acid is a monocarboxylic acid with a slightly pungent, disagreeable odor. Salts of the acid have a slight cheeselike flavor. The acid form is readily miscible with water, and the sodium salt is more soluble than the calcium form. It is normally found in cheese and as a metabolite in the ruminant gastrointestinal tract. Swiss cheese contains up to 1% propionic acid because of the growth and metabolism of propionibacteria, which are associated with its manufacture and the characteristic Swiss cheese flavor. This naturally formed additive becomes a developed preservative that limits mold growth on Swiss cheese. This additive is also used as a mold inhibitor in cheese foods and spreads. Its antimicrobial effect is limited to most yeast and bacteria.

Propionic acid and its salts are used primarily as mold and rope inhibitors in bread. Baking destroys most molds, but surface recontamination can occur during packaging, and growth can be seen under the wrapper during storage. Propionates can be added to bread dough without interfering with leavening because there is little or no effect on yeast. Sodium propionate is recommended for use in chemically leavened products because the calcium ion interferes with the leavening action. Calcium propionate is preferred, however, for use in bread and rolls because the calcium contributes to the enrichment of the product (Chichester and Tanner, 1972).

Propionic acid (21 CFR 184.1081) and its salts, calcium (21 CFR 184.1221) and sodium propionate (21 CFR 184.1784), are approved as GRAS substances for miscellaneous and general-purpose usage. In addition, calcium and sodium propionate are listed as antimycotics when migrating from food-packaging material (21 CFR 181.23). No upper limits are prescribed for use of this additive, except bread and rolls, which conform to standards of identity. A limit of 0.32% can be used in flour and in white bread and rolls, 0.38% in whole-wheat products, and 0.3% in cheese products (Robach, 1980). The acceptable daily intake for humans is listed in Table 4.3.

SUCCINIC ACID

Antimicrobial Properties

Succinic acid successfully lowered microbial loads on poultry carcasses (Mountney and O'Malley, 1965); however a 3% or 5% concentration used at 60°C impaired the appearance of the product (Cox et al., 1974). Thomson et al. (1967) reduced the population of inoculated *S. Typhimurium* by 60% on chicken carcasses after spraying with 1.0% succinic acid.

Toxicology

In short-term studies, rats received subcutaneous injections of 0.5 mg succinic acid daily, gradually increasing to 2.0 mg/day at 4 weeks and continuing at this level for 100 days. The test animals did not differ significantly in reproduction rates, hair appearance, tooth eruption, or eye opening from the control group. Chick embryos developed normally when comparable dosages were injected into the air sac (Dye et al., 1944).

Application and Regulatory Status

Succinic acid is a nonhygroscopic, dicarboxylic acid having low water solubility. It has a slightly bitter taste and serves as a flavor enhancer. Succinic acid is used to modify the plasticity of bread doughs and in the production of edible fats. It can be incorporated into gelatin deserts and cake flavorings (Gardner, 1972). Succinic acid is approved as a GRAS substance for miscellaneous and general-purpose usage (21 CFR 184.1091). No limit has been set on the acceptable daily intake for humans.

TARTARIC ACID

Antimicrobial Properties

Any antimicrobial property of tartaric acid is because of its ability to lower the pH.

Toxicology

Acute toxicity data for tartaric acid by the intravenous route for mice indicates an LD₅₀ of 485 mg/kg body weight (Table 4.2). Rose's (1924) work on subcutaneous administration of 0.25 to 1 g to rabbits produced abnormalities in blood chemistry, including increased nonprotein nitrogen, sugar, and cholesterol levels. However, male rabbits consuming a diet containing 7.7% sodium tartrate (5% tartaric acid) did not show any changes in food consumption, growth, mortality, or gross histology compared with controls (Packman et al., 1963). Fitzhugh and Nelson (1947) found similar results in long-term studies using 21-day-old weanling rats fed tartaric acid as 0.1%, 0.5%, 0.8%, and 1.2% of the diet.

Studies measuring teratogenic activity in pregnant mice, rats, hamsters, and rabbits demonstrated normal offspring at levels of 274, 181, 225, and 215 mg/kg body weight, respectively (Food and Drug Research Laboratory, 1973a). Workers exposed to tartaric acid dust measuring up to 32 mg/m³ in a manufacturing plant that produced the acid experienced tooth erosion. The workers also displayed skin eruptions, which disappeared after they left the work site (Barsotti et al., 1954).

Application and Regulatory Status

Tartaric acid, the most soluble of the solid acidulants, has a strong tart taste that enhances grapelike flavors. It is used in fruit jams, jellies and preserves, sherbets, and grape-flavored beverages. The monopotassium salt (cream of tartar) is commonly found in baking powders. Tartaric acid acts synergistically with antioxidants to prevent rancidity, and it prevents discoloration in cheese (Gardner, 1972). Tartaric acid is a GRAS substance for miscellaneous and general-purpose usage in accordance with good manufacturing practices (21 CFR 184.1099). The acceptable daily intake for humans is listed in Table 4.3.

MODE OF ACTION

The mode of action of organic acids in inhibiting microbial growth appears to be related to maintenance of acid–base equilibrium, proton donation, and the production of energy by the cells. It is thus essential to understand each of these concepts before consideration of the actual mode of action of the organic acids. Biological and chemical systems depend on an interaction between acid and base systems. The microbial cell normally reflects this equilibrium by attempting to maintain an internal pH near neutrality (Baird-Parker, 1980). Homeostasis is the tendency of a cell to sustain chemical equilibrium despite a fluctuation in the acid–base environment. Through the interaction of a series of chemical mechanisms, this delicate balance is maintained and alteration of this balance causes destruction of the microbial cell. Proteins, nucleic acids, and phospholipids can be structurally altered by pH changes. The availability of metallic ions to the organism is also altered and becomes a function of membrane permeability because membranes are less permeable to charged molecules than to uncharged molecules. These changes in membrane permeability can exert a dual effect by impairing transport of nutrients into the cell or by causing the leakage of internal metabolites to the outside. Changes resulting from pH can destroy bacteria, molds, and yeasts, although some microorganisms such as *Acetobacter* can exist at and even require extremes of acidity (Langworthy, 1978).

The terms strong or weak are used to describe acids and reflect the degree with which acids readily donate a proton or dissociate in aqueous solutions. Inorganic acids, such as hydrochloric,

because of their low pKa almost entirely dissociate in solution. Acetic acid and other organic acids only slightly ionize and do not readily give up their proton(s) to water. When an acid enters and ionizes within the cell, the problem becomes one of elimination of the excess protons. This ejection process becomes a fundamental issue in how a cell produces energy.

Energy produced through chemical reactions is essential for cellular processes. Synthesis of macromolecules, maintenance of osmotic gradients, and active transport of molecules across the membrane depend on energy generated by the cell in the form of adenosine triphosphate (ATP). In anaerobic microorganisms, the glycolytic pathways inefficiently generate ATP. In aerobic organisms, the electron transport system (ETS) principally generates ATP with oxygen as the terminal electron acceptor (Gould et al., 1983).

Before energy can be produced, materials must be transported into the cell. One means for substrate entry into the cell is active transport, which also allows greater internal concentration of solute than external concentration. To maintain this unequal gradient, energy must be expended. For this reason, active transport is coupled with energy-yielding processes. The energy evolves from the oxidation of the substrates and the respiratory chain. The respiratory chain or ETS transverses the membrane, which does not permit H⁺ or OH⁻ ions to penetrate and ejects accumulated protons to the external environment. This process generates a chemical and electric gradient capable of driving metabolic reactions (Dawes and Sutherland, 1992).

Early experiments by Levine and Fellers (1940) demonstrated that acetic acid was more lethal at a higher pH than hydrochloric acid or lactic acid. They concluded that this toxicity was not the result of hydrogen ion concentration alone, but seemed to be a function of the undissociated molecule. With acetic acid, lowering the pH increased the inhibitory activity, confirming that the undissociated molecule was the effective inhibitor. Thus, the inhibition by extracellular fatty acids used as antimicrobial agents would increase with decreasing pH, in agreement with pKa values (Freese et al., 1973).

Sheu and Freese (1972) determined that short-chain fatty acids reversibly reacted with the cell membrane and altered its structure. It was postulated that this interfered with the regeneration of ATP by uncoupling the ETS, or the transport of metabolites into the cell was altered. Further studies by Sheu and coworkers (1972) indicated that acetate uncoupled the amino acid carrier protein from the ETS and inhibited amino acid transport noncompetitively. Serine uptake was inhibited in membrane vesicles of *B. subtilis* when exposed to fatty acids; using the same system, L-leucine and L-malate were shown to uncouple both substrate transport and oxidative phosphorylation from the ETS (Freese et al., 1973).

In a later study, Sheu et al. (1975) recognized that if reducing compounds were no longer available to the cell because of transport inhibition by the fatty acids, oxygen consumption would be reduced. To understand these processes, the bacterial cell was converted to a spheroplast under isotonic conditions and these membrane vesicles were used to study uptake of a substrate against a gradient. Because oxygen consumption was observed in the presence of membrane preparations and an energy source, inhibition transport would not necessarily be linked with ATP generation. The active transport of a molecule would depend on the proton gradient generated during the oxidation of a substrate. Lipophilic agents, such as the short-chain fatty acids, would shuttle protons through the membrane until the proton motive force had been destroyed and transport thus eliminated.

Freese and Levine (1978) further postulated that the most effective antimicrobial agents would be lipophilic enough to attach to microbial membranes yet be soluble in the aqueous phase. This is because they can approach the membrane from the aqueous medium, yet easily and without requiring energy penetrate the membrane lipid bilayer. Undissociated acids of short chain length can penetrate the cell more easily because they possess these characteristics.

Not all acids are effective against all microorganisms. *E. coli* and *B. subtilis* are equally inhibited by equal concentrations of compounds containing up to six carbons. However, twice the amount of a C8 (caprylic) acid is needed to inhibit the growth of *E. coli* compared to *B. subtilis*. The

difference between the two organisms could be because of the lipopolysaccharide layer that surrounds the Gram-negative *E. coli*, thus acting as a mechanical barrier and preventing passage of the acid into the cell. Another theory proposes that Gram-negative organisms can rapidly metabolize the acidulant and therefore not allow it to accumulate within the cell (Freese et al., 1973; Kabara et al., 1972). Furthermore, some acids, lactic and citric, reduced the internal pH of the cell more than acetic acid; however, other changes associated with metabolic or physiologic processes may be similarly affected (Ita and Hutkins, 1991).

Hunter and Segal (1973) in studies using *Penicillium chrysogenum* suggest that weak acids at or below their pKa could discharge the proton gradient and ionize within the cell to acidify the interior. It was postulated that the rate of proton leakage into the cell versus proton ejection would determine the inhibition of the cell (Freese et al., 1973). Eklund (1980) has questioned the mode of action of the weak lipophilic acids. He states that although inhibition of uptake of nutrients contributes to growth inhibition, it does not seem to be the sole cause of the static action.

In summary, the current data suggest that the mode of action of short-chain lipophilic acids requires the destruction of the proton motive force, thereby limiting substrate transport. It is further speculated that acids, which possess both lipoidal and aqueous solubilities, are the most effective antimicrobial agents and that some sort of membrane attachment of the acid is involved. Studies have indicated that cells, when placed in fresh medium devoid of the inhibitor, can reinstate transport, suggesting a static response rather than a killing effect (Freese et al., 1973). Obviously, there is need for further research on the mode of action of these antimicrobial agents.

ACID ADAPTATION AND RESISTANCE

Predictable response of cells to stressful situations is the cornerstone of the multiple-hurdle concept, whereby organisms are subjected to multiple deleterious compounds or processes designed to reduce or eliminate microbial loads. These stresses occur simultaneously, such as the addition of several antimicrobial agents (acid, bacteriocin, preservative) to a food, or sequentially, such as intervention strategies that occur during processing of muscle foods (acid sprays, hot water rinses). In sequential treatments, there may be more of an opportunity for strains to develop resistances to the antimicrobial compound or treatment provided that time of exposure allows for such development.

Cells strive to maintain a balance between competing stresses that occur through exposure to sublethal conditions, namely, acidity, temperature, available water, starvation, and desiccation, among others. Generally, organisms survive and function over a wide range of acidity and alkalinity as a result of their ability to maintain homeostasis; however, exposure to adverse environments frequently induces a stress response in susceptible microorganisms. Response to such stresses often takes the form of specific stress proteins induced after sublethal exposure. It is hypothesized that these proteins protect against subsequent exposure to the same stress or possibly cross protect against other stresses (Davidson and Harrison, 2002). Adaptation to sublethal stresses allows organisms to adapt to higher levels of the same stress without being killed. For example, exposure to low concentrations of organic acids would cause organisms to become more resistant to higher concentrations of acids, thus potentially allowing them to survive exposure to commonly used antimicrobial intervention strategies. Depending on the length of time and exposure to mild acid conditions, cells can adapt by means of an acid tolerance response. Exposure to greater quantities of acid (lower pH levels) causes acid shock, which can lead to cell death. During the shock period, stress-induced proteins are formed, which can be specific to the type of shock conditions.

Adaptive responses to sublethal exposure to acids can often offer cross protection to cells when exposed to other stresses. Cross-protective effects have been demonstrated in *E. coli* between initial exposure to organic acids and subsequent exposure to salt (Garren et al., 1998; Jordan and Davies, 2001), radiation (Buchanan et al., 1999), and heating (Mazzotta, 2001; Ryu and Beuchat, 1998). The adaptive effect has also translated to survival of microorganisms in acidic foods, such as *E. coli* O157:H7 in salami and apple cider (Leyer et al., 1995), *S. Typhimurium* in cheeses (Leyer and

Johnson, 1992), and *L. monocytogenes* in fermented dairy products (Gahan et al., 1996). In addition, cell survival is affected by its previous growth environment, including oxygen conditions, growth phase, and temperature (Cheng and Kaspar, 1998). Conversely, pretreatment with acids increased sensitivity of *S. Typhimurium* to chlorine and iodine (Leyer and Johnson, 1997), leading to potentially more effective interventions.

Measurement of survival or injury of organisms subjected to stresses is dependent on the methods designed to recover organisms. It is assumed that inhibitory compounds that affect optimal recovery of cells would be avoided. It is interesting that higher survival levels were achieved in acid-injured *E. coli* when dilutions were made in osmotically stable diluents containing sucrose, sorbitol, glucose, or sodium chloride, but not glycerol (Jordan et al., 1999).

Acid adaptation is also seen in Gram-positive organisms, such as *L. monocytogenes* (Kroll and Patchett, 1992). Preexposure to mild acid conditions led to increased resistance to higher acid environments such as those encountered in the stomach or macrophage phagosome (O'Driscoll et al., 1996).

Inducement of stress conditions can result in the shortening of processing systems and can optimize the use of multiple hurdles as a preservation process (Brul and Coote, 1999; Brul et al., 2002). Using acid-adapted strains of salmonellae inoculated into lean beef tissue, Dickson and Kunduru (1995) demonstrated that exposure to organic acid rinses did not lead to more resistant organisms. It was further shown (Uyttendaele et al., 2001) that acid-resistant, acid-sensitive, and acid-inducible strains of *E. coli* O157:H7 presented no difference in their survival when inoculated into beef tissue treated with 1% and 2% buffered lactic acid.

Shadbolt et al. (2001) postulated that exposure of *E. coli* to stresses such as low water activity (0.90) or low pH (3.5) could produce a biphasic death phase. This was readily apparent if the first stress was low water activity. If the stresses were in reverse order, pH rapidly inactivated cells such that a biphasic death phase was not observed. The authors hypothesized that disruption of cell homeostasis created a large energy drain, thus sensitizing cells to future environmental stresses.

ASSAY

The various acids can be assayed by relatively simple procedures. Methods for determining acid content in foods are found in the *Official Methods of Analysis of the Association of Analytical Chemists* (Horowitz, 2000) or *Handbook of Food Analysis* (Nollet, 1996).

Volatile acids can be separated from foods using steam distillation. If only one acid is present, the distillate can be titrated for the particular acid. For a mixture of acids, the distillate is separated on a silicic acid column and the component acids can be identified using enzymatic, chromatographic (gas, paper, thin-layer, high-performance liquid chromatography), or electrochemical methods. Qualitative or quantitative methods can be used depending on the degree of sensitivity required. Often derivatives of the acids are required, which adds to sample preparation time (Gomis and Alonso, 1996).

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5 Sulfur Dioxide and Sulfites

Cornelius S. Ough and Lilian Were

CONTENTS

Introduction	143
Source.....	143
Form and Solubility	144
Reactivity.....	145
Antimicrobial Activity	148
Bacteria	148
Yeast	151
Molds.....	154
Other Uses.....	155
Toxicology.....	155
Amounts in Foods and Regulations.....	157
Analytical Methods	158
References	160

INTRODUCTION

It was quoted by Hammond and Carr (1976) that the poet Homer burned sulfur to disinfect his home in Greece around the 8th century B.C. From that time and perhaps even before, sulfur dioxide (SO₂) has been used as an antiseptic, disinfectant, or sanitizer. In its various forms as salts, dissolved in water, or as a gas, it is used in fermentations, on fruit, and in other industries to prevent microbial activity or growth. It is especially important in the production of wine. General reviews of the use of sulfites in wine and their effect on yeasts include Schopfer and Aerny (1985), Herraiz and Cabezudo (1989), Ough and Crowell (1987), and Bakalinsky (1992). Other uses for sulfites are as an antioxidant, to inhibit enzymatic browning inhibitor and the Maillard reaction, as a dough conditioner, and to prevent black spot on crustaceans (Papazian, 1996; Gould, 2000).

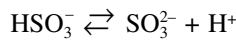
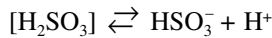
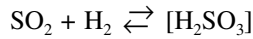
SOURCE

Sulfur, sometimes called brimstone, occurs in the free state in some regions of the world. Sulfur oxide is especially common to the volcanic areas. Sulfites and sulfates are also found in nature. Sulfur burns in the presence of air to give sulfur dioxide (McAlpine and Soule, 1933), which is a colorless gas with an extremely suffocating odor. It is fairly soluble in water, existing mainly as sulfur dioxide molecules, with some molecules associated with water. According to Schroeter (1966), the monohydrate H₂SO₃ does not exist. Sulfur dioxide gas can be easily liquefied by compression. At 20°C it has a vapor pressure of 329 kPa (3.25 atm).

Sulfur dioxide can be prepared commercially from burning sulfur (oxidation), heating pyrites, and from reducing gypsum, as well as by other methods. The anhydrous sodium and potassium salts are prepared by precipitation and then dehydrated with the appropriate hydroxide. Most of these salts are hygroscopic and easily hydrolyzed (Schroeter, 1966).

FORM AND SOLUBILITY

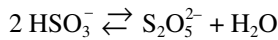
In water solutions, sulfur dioxide can be written to show the equilibrium:



The bracketed form indicates the sulfur dioxide associated with water.

The pK_a values for sulfur dioxide are 1.76 and 7.20, indicating a rather weak dibasic acid (Segal, 1968). A plot of the distribution of the three ionic forms can be calculated and is shown in Figure 5.1. Specific gravity for various water solutions of sulfur dioxide is shown in Table 5.1.

It is useful to have the sulfur dioxide in a salt form. The dry salts are easier to store and are less of a problem to handle than gaseous or liquid sulfur dioxide. Table 5.2 lists the main forms of sulfur dioxide, their theoretical yields, and solubilities of each in water. The metabisulfite is the anhydride of the acid sulfite:



When these salts are exposed to air, they show increasing stability in the order sulfite > bisulfite > metabisulfite (Mason, 1928; Phillips, 1928).

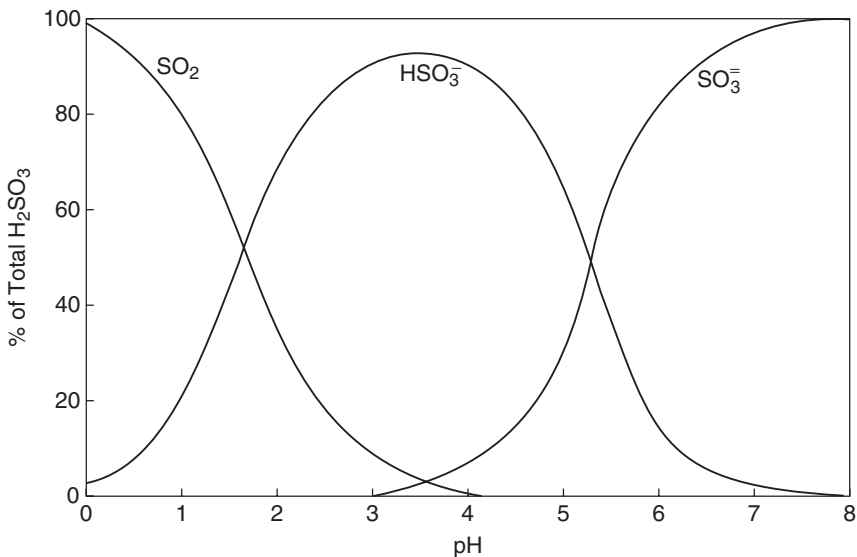


FIGURE 5.1 Distribution of the ionized forms of sulfurous acid at various pHs.

TABLE 5.1
Specific Gravity of Various Sulfur Dioxide Water
Solutions at Two Temperatures

SO ₂ (g per 100 g)	Specific Gravity ^a	
	15.56°C (60°F)	20°C (68°F)
1	1.0040	1.003
2	1.0091	1.008
3	1.0191	1.018
6	1.0292	1.028
8	1.0393	1.037
10	1.0493	—

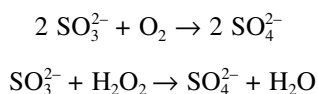
^a Corrections are approximately 0.0001°F⁻¹

TABLE 5.2
Sulfur Dioxide-Bearing Chemicals

Compound	Formula	Theoretical Yield (%)	H ₂ O Solubility (g/L)
Sulfur dioxide	SO ₂	100	110 (20°C)
Potassium sulfite	K ₂ SO ₃	33	250 (20°C)
Sodium sulfite	Na ₂ SO ₃	50.8	280 (40°C)
Sodium sulfite heptahydrate	Na ₂ SO ₃ ·7H ₂ O	25.4	240 (25°C)
Potassium bisulfite	KHSO ₃	53.5	1000 (20°C)
Sodium bisulfite	NaHSO ₃	61.6	3000 (20°C)
Potassium metabisulfite	K ₂ S ₂ O ₅	57.6	250 (0°C)
Sodium metabisulfite	Na ₂ S ₂ O ₅	67.4	540 (20°C)

REACTIVITY

Wedzicha (1984) briefly reviewed the chemical interactions of sulfur dioxide. The oxidizability of the sulfurous acid salts is indicated by the following equations:



Jacobs (1976) showed that the amount of sulfur dioxide that reacted (oxidized) after 60 days of storage in bottled red wine was proportional to the original dissolved oxygen content of the wine. After the oxygen disappeared, much slower changes occurred in the sulfur dioxide content.

If ascorbic acid is being used in combination with sulfur dioxide, the second equation listed previously is a key reaction (Heinmann et al., 1970). The sulfur dioxide scavenges the hydrogen peroxide formed and keeps further oxidation of the dehydroascorbic acid and other products to a minimum. This reaction is extremely rapid. Holt and Kumar (1986) found an observed $k = 41.7 \pm 3.4 \text{ s}^{-1}$ for H₂O₂ with SO₃²⁻ at pH 3.40 and 15°C. The sulfur dioxide stabilizes the dehydroascorbic acid by reacting with the ketone bonds (Wisser et al., 1970).

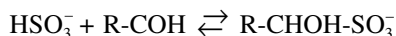
TABLE 5.3
Relative Percentage of Aldehydes that React with Sulfur Dioxide
and Their Equilibrium Constants (K)

Compound	1	2	3	K ^a
Acetaldehyde	100	99.5	50	1.5×10^{-6}
Pyruvic acid	66	72	32	3×10^{-4}
α -Ketoglutaric acid	47	44	18	5×10^{-4}
Glyoxylic acid	—	98	—	8×10^{-6}
L-Xylosone	27	—	—	—
Oxaloacetic acid	—	66	—	2×10^{-4}
Glucuronic Acid	—	1	—	5×10^{-2}
Monogalacturonic Acid	2.5	2	—	1.7×10^2
Rhamnose	—	—	26	—
Trigalacturonic Acid	2.1	—	—	—
Arabinose	—	—	45	—
Xylose	1.1	—	32	—
Fructose	—	0.1	0.6	15.0
Glucose	0.12	0.1	7.7	0.9
Malvidin-3-glucoside	—	87	90	6×10^{-5}

Note: 1 = Burroughs and Whiting (1960); 2 = Aerny (1986a,b); 3 = Navara (1985).

^a Aerny (1986 a,b).

Bisulfite addition products, the hydroxysulfonic acids (Suter, 1944), form rapidly with aldehydes:



All aldehydes form the hydroxysulfonates, but not all ketones react. Diethyl ketone reacts slowly and to a limited extent. Otherwise, only ketones with a methyl group adjacent to the carbonyl or carbonyls that are part of a four- to seven-member carbon ring system will react. Reactions with the sugars are limited to those with a free aldehyde and are much slower and the products are less stable (Gehman and Osman, 1954; Joslyn and Braverman, 1954). Ingram and Vas (1950) found that galactose, mannose, and arabinose reacted rapidly with bisulfite; maltose, lactose, and glucose reacted less rapidly; raffinose reacted very slowly; and fructose and sucrose did not react at all. Those reacting the most rapidly formed complexes that dissociated the least. Relative percentages of aldehydes reacting with sulfur dioxide and their equilibrium constants are shown in Table 5.3 (Burroughs and Whiting, 1960; Aerny, 1986 a,b; Navara, 1985).

In moldy apples, fermented ciders, and wines, 2,5-diketogluconic acid, 2-oxogluconic acid, 5-oxofructose, L-xylosone, d-threo-2,5-hexodiulose, acetaldehyde, pyruvate, α -ketoglutarate, and galacturonic acid were significant bisulfite binding forces (Burroughs and Sparks, 1964a, 1973; Lea et al., 2000). In botrytized grapes made into wine, as much as 80% of the total sulfur dioxide may be bound by these types of carbonyls (Blouin, 1963). Rhem (1964) noted that the rate of formation and the amount of sulfonate formed depended on the concentration of the reactive substances, the pH, and the temperature. He also noted that phosphoglyceraldehyde would likely react.

Glucose is by far the most abundant of the reactive aldehydes and ketones in most fruit juices. Compared with model solutions, natural fruit juices always bind more sulfur dioxide than would be calculated from the glucose present in the juice (Joslyn and Braverman, 1954).

The amounts of aldehydes, ketones, and other SO₂ binding substances limit the effective use of the added sulfite. Lafon-Lafourcade (1985) reviewed the role of yeast and bacteria in the amounts of these components found in wines. Farris et al. (1983) tested 30 strains of *Saccharomyces cerevisiae* for production of SO₂ binding materials. They measured acetaldehyde, pyruvate, and α-ketoglutarate. The respective ranges and means for these components were 72 to 287 and 115.5 mg/L, 16 to 42 and 27.5 mg/L, and 15 to 57 and 31.6 mg/L. Farris et al. (1982), from winery trials, selected four yeasts that produced low levels of SO₂ binding compounds.

Farkaš et al. (1985) found a reduction in the content of the SO₂ binding materials by the addition of 0.5 mg/L of thiamine. This treatment allows more effective use of SO₂ in wine. Piracci and Spera (1983) reported similar results. Uzuka et al. (1984) also showed that sulfite additions increased acetaldehyde, but they found no increase in pyruvate or α-ketoglutarate.

Bisulfites may also react with nucleotides such as nicotinamide adenine dinucleotide (Meyerhof et al., 1938). Shapiro and Weisgras (1970) also demonstrated that cytosine was transformed to cytidine by bisulfite. There is no evidence that these reactions occur *in vivo*.

Damant et al. (1989) found a food-coloring dye, sunset yellow, reacted with bisulfite to form a lemon yellow compound. The addition product attached at the carbon 4 of the sunset yellow molecule. Traces of the compound could be found in stored commercial soft drinks that had been heated and contained sulfite.

Sulfur dioxide can loosely bind to anthocyanins. Jurd (1972) suggested the binding site for HSO₃⁻ is on the 4-position rather than the 2-position (e.g., malvidin monoglucoside). This contributes to the difficulty in the measurement of free sulfur dioxide in highly colored wines. Glories (1984) studied the equilibrium between SO₂ and the anthocyanin–bisulfite complex. In model solutions in the range of 30 to 50 mg/L of SO₂ added, the following formula was used to calculate the equilibrium constant K_s .

$$K_s = [\text{AHSO}_3]/([\text{A}^+][\text{S}] - (\text{AHSO}_3^-)) = 10^5\text{M}^{-1}$$

where:

- A = total anthocyanins
- A⁺ = anthocyanins in the ionized form (colored)
- AHSO₃ = anthocyanin–bisulfite complex
- S = HSO₃⁻ added (calculated from Henderson-Hasselbach equation)

At 10 mg/L of SO₂ added, the color is reduced by about 25%, and at 50 mg/L of SO₂ added, it is reduced by 80%.

Heintz (1976) reported the occurrence of an addition product of sorbic acid and bisulfite. After approximately 100 days of storage, a potassium bisulfite solution (148 mg/L) with sorbic acid (200 mg/L) contained 62% less sulfur dioxide compared to a standard solution of potassium bisulfite with no added sorbic acid. This could be significant in wine because the reduction of free sulfur dioxide could result in the malolactic bacteria being available to act on the remaining sorbic acid to form 2-ethoxyhexa-3,5-diene (Crowell and Guymon, 1975).

Allyl isothiocyanate in mustard was shown to react with sulfites used as antioxidants to form allylaminothiocarbonyl sulfonate (Cejpek et al., 1998). The reaction affected flavor of the mustard by reducing pungency.

Undoubtedly other reactions with sulfur dioxide take place that could reduce the amounts of available sulfur dioxide, but these have been reported as the primary reactions.

Margheri et al. (1986) and Bach and Hess (1983) could not find any correlation between amino acid levels in the medium and the accumulation of SO₂ binding compounds. Dittrich and Barth (1984) did find correlations between the SO₂ binding substances and wineries and grape source.

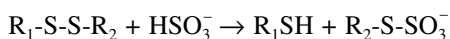
They analyzed 544 German wines. Of the three major SO₂ binding components, pyruvate was always found in the smallest amounts. Somers and Wescombe (1987) noted that wines that had undergone malolactic fermentation decreased significantly in the SO₂ binding components, with a corresponding increase in free sulfite.

ANTIMICROBIAL ACTIVITY

The growth-inhibiting or lethal effects of sulfurous acid are most intense when the acid is in the un-ionized form (Hailer, 1911). It has also been noted that bacteria are much more sensitive to sulfur dioxide than are yeasts and molds. Schelhorn (1951) observed that bisulfites had lower activity than sulfur dioxide against yeast, and the sulfites had none.

The bound forms of sulfur generally have reduced antimicrobial activity (Rhem, 1964; Schroeter, 1966). Cruess (1912) estimated that, in grape juice, the bound forms of sulfurous acid had about 1/30 the antimicrobial effectiveness of the free form. Although sulfonates have decreased antimicrobial activity, several have been found to inhibit yeast respiration (Rhem, 1964). The order of decreasing antimicrobial activity of the sulfonates was pyruvate > benzaldehyde > arabinose > ketoglutarate > acetone > acetaldehyde > glucose > fructose (Rhem, 1964).

Another reaction of significance is that between bisulfite and disulfide bonds:



This reaction can cause conformational changes in enzymes.

Thiamine pyrophosphate, a required cofactor for many enzymatic reactions, can be destroyed by the action of bisulfite (Williams et al., 1935). Excess sulfur dioxide added to grape juice can deplete thiamine and inhibit fermentation (Ournac, 1969).

One type of activity of sulfite against the yeast cell is its reaction with cellular adenosine triphosphate (ATP) (Schimz and Holzer, 1977, 1979; Schimz, 1980) and/or its blocking of the cystine disulfide linkages. Hinze et al. (1981) also found reduced ATP activity by addition of SO₂ to lactic acid bacteria. Anacleto and van Uden (1982) suggested that the antimicrobial effects of SO₂ occurred at the surface of the cell. They proposed two receptor sites. One site was directly related to the death process. The other modulated the entropy of activation of the process. The cytoplasmic membrane has a high affinity for reaction with SO₂. Beech and Thomas (1985) give an excellent review of the many antimicrobial actions possible with SO₂. Among the activities discussed are blockage of transport, inhibition of glycolysis, nutrient destruction, and inhibition of general metabolism.

Lenz and Holzer (1985) showed that the depletion of thiamine pyrophosphate (TPP) in *Saccharomyces cerevisiae* by SO₂ at levels used for juice and wine preservation caused the TPP-dependent enzymes to decrease in activity. Pyruvate decarboxylase and transketolase lost 42% and 87% of their activity, respectively. TPP activity losses were slower than ATP losses, but the overall losses were about the same.

When considering the antimicrobial activity of sulfur dioxide and its salts, three main groups of microbes are of interest in the acid beverages and fruits. These are as follows: (1) acetic acid-producing and lactic acid-producing bacteria, (2) fermentation and spoilage yeasts, and (3) fruit molds. Sulfites are used in other foods and pharmaceuticals, but their major use as an antimicrobial agent is in beverages and fruits.

BACTERIA

According to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994), the genus *Acetobacter* is Gram-negative aerobic rods. The type species is *A. aceti*. These bacteria are able to

oxidize ethanol in fermented beverages to acetic acid and, further, to carbon dioxide and water. They are aerophilic and have pH optimum for growth of around 5.4 (Holt et al., 1994).

Dupuy (1959) postulated that the reversible reaction between sulfur dioxide and cysteine to form thiol esters along with thiamine and NAD⁺ degradation were the causes for inhibition of *Acetobacter*. He found that this genus also fixed a certain amount of the sulfur dioxide. Cruess (1912) found that 5.49 log *Acetobacter* per ml exposed to 100 and 200 mg/L of total sulfur dioxide in grape juice were reduced to 2.47 log and 0.3 log CFU/ml, respectively, after 36 hours. Rhem and Wittmann (1962) reported that 200 mg/L sulfur dioxide killed *Acetobacter* at pH 6.0 in a buffered solution. Dupuy and Maugenet (1963) noted that even small doses of sulfur dioxide inhibited the activity of the cells, but much larger doses were required for bactericidal action. Lafon-Lafourcade and Joyeux (1981) and Joyeux et al. (1984) stated that the amounts of SO₂ used in normal wine making are insufficient for acetic acid bacteria control. They indicated *A. aceti* can grow in red wine with 25 mg/L of unbound sulfur dioxide present. Watanabe and Ino (1984) and Juven and Shomen (1985) reported that up to 100 mg/L of total sulfites for grape juice, red wine, and soft drinks were required to control acetic acid bacteria.

Several reports (Károva and Kircheva, 1982; Spirov et al., 1983) indicated that around 50 mg/L of free sulfite could preserve wine vinegar for about half a year. Cell growth was completely prevented. Additions of 100 mg/L bleached the color of the vinegar.

Bacteria common in acid fruits and beverages are the lactic acid-producing genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus*. The homolactic species found in wines are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus leichmannii*, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, and *Pediococcus pentosaceus*, and the heterolactic species are *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus hilgardii*, *Lactobacillus trichodes*, and *Oenococcus oeni* (Amerine et al., 1980). *Leuconostoc mesenteroides* subspecies *dextranicum* can also cause ropiness in beverages through the formation of dextrans. Fornachon (1957) noted that sulfur dioxide and pH were important factors in controlling lactic bacteria in wines. Levels above 120 mg/L of total sulfur dioxide (free and bound) decreased the incidence of malolactic fermentation, with lower pH increasing the effectiveness of the sulfur dioxide. Amerine et al. (1980) reported that 30 mg/L of free sulfur dioxide is sufficient to inhibit malolactic fermentation in wine. In model solutions containing *Lactobacillus arabinosus* and *L. casei*, 320 and 651 mg/L of sulfurous acid were required, respectively, for antimicrobial action at pH 6.0 (Rhem and Wittmann, 1962).

Carr and Davies (1971) noted that relatively high amounts of free sulfurous acid were present in ciders that contained viable lactobacilli. They found that up to 1.5 mg SO₂ per liter in the undissociated form was bacteriostatic to *L. plantarum*. Concentrations above 1.5 mg/L were bactericidal. *Lactobacillus trichodes* (Fornachon et al., 1949) is described as causing a hairlike growth in fortified wines. This organism is particularly intolerant to sulfur dioxide. As low as 75 to 80 mg/L of total sulfur dioxide prevents growth and 100 mg/L kills this species. The bacterium, however, is very heat resistant and alcohol tolerant. Lafon-Lafourcade (1975) demonstrated the effectiveness of both free sulfur dioxide and bound sulfur dioxide in inhibiting *Leuconostoc gracile* in wine at pH 3.5. Addition of 30 mg/L of free sulfur dioxide killed the bacteria completely in 15 days; 20 days was required to kill the bacteria when the same amount of aldehyde–bisulfite complex was used.

Oenococcus oeni grows preferentially at lower pH but seems less tolerant to sulfur dioxide (Mayer, 1979). As little as 30 mg/L of added sulfur dioxide may be lethal to the species. Manca de Narda and Strosser de Saad (1987) investigated the tolerance of *O. oeni*, *L. hilgardii*, and *P. pentosaceus* to SO₂, pH, and ethanol. *O. oeni* was the most sensitive to SO₂.

Fermentation of grape juices with larger amounts of insoluble solids present resulted in wines that underwent malolactic fermentation sooner and more rapidly than those juices that contained fewer solids (Liu and Gallander, 1982, 1983). The juices high in insoluble solids ultimately had lower residual total sulfites. Liu and Gallander (1983) demonstrated that lower pH wines underwent

malolactic fermentation more slowly and that lower sulfur dioxide levels increased the fermentation rate of the *O. oeni* PSU-1 used. Ough et al. (1988) demonstrated that without sufficient SO₂ and adjusted pH, the growth of *O. oeni* in red table wine occurs readily. There was some additive effect when used with dimethyl dicarbonate but not enough to warrant the use with SO₂ for this purpose. Piracci (1984) found 0.5 mg/L of molecular sulfite was sufficient to control malolactic bacteria growth.

Lafon-Lafourcade et al. (1983) determined that, in the Bordeaux area of France, *O. oeni* was the primary malolactic bacterium associated with wine. It survived alcoholic fermentation when others did not. It was found to tolerate up to 100 mg/L of SO₂, although after sulfite addition, a rapid decline in cell numbers occurred. The remaining cells later multiplied to significant numbers. Wibowo et al. (1988) found significant delays in growth of *O. oeni* when SO₂ was added and quite a variable response between strains. Davis et al. (1988) tested 146 different strains of wild malolactic bacteria. All strains grew at pH 4.5 in beef broth and 20% tomato juice serum medium at 64 mg/L of total sulfur dioxide. *O. oeni* strains were less tolerant to the sulfur dioxide than *Pediococcus parvulus* strains or the *Lactobacillus* species. This work confirms that wines with high total sulfur dioxide concentrations are more likely to undergo malolactic fermentation with other than *O. oeni* strains with unfavorable sensory results.

Splittstoesser and Stoyla (1989) looked at five different regulatory-approved additives to determine if they could suppress malolactic bacterial growth in grape juice as a replacement for sulfites. None of the compounds alone, or in paired combinations, were completely effective.

Millet and Lonvaud-Funel (2000) reported that sulfites cause a portion of lactic acid and acetic acid bacterial populations to enter a viable but nonculturable (VBNC) state. The cells could not be cultured on nutrient agar plates but demonstrated metabolic activity through hydrolysis of fluorescent esters and were countable using direct epifluorescence microscopy. They suggested that these microorganisms could cause spoilage in wines that were considered sterile using conventional counting techniques.

Roberts and McWeeny (1972), in their review, state that sulfur dioxide is more effective against the growth of Gram-negative rods, such as *Escherichia coli* and *Pseudomonas*, than in inhibiting Gram-positive bacteria. This is demonstrated in the use of sulfites in meats. The preservation of the color and odor of meats is improved by sulfite treatment and, although slowing or prevention of growth of surface bacteria is probably important, the main effect in meat appears to be the antioxidant properties (Roberts and McWeeny, 1972). Banks et al. (1985) reviewed the use of sulfite as an additive to control microbiological changes occurring in meat products. They noted that sulfites shifted the microflora of the meat to Gram-positive bacteria from the normal Gram-negative flora. The Gram-positive bacteria remaining grew more slowly than the Gram-negative bacteria. In addition, *Salmonella* and *E. coli* were inhibited to a greater extent by sulfites than other bacteria. Banks and Board (1982) tested several genera of Enterobacteriaceae isolated from sausage for their metabisulfite sensitivity. The microorganisms tested and the concentration of free sulfite (µg/ml) necessary to inhibit their growth at pH 7.0 were as follows: *Salmonella*, 15–109; *E. coli*, 50–195; *Citrobacter freundii*, 65–136; *Yersinia enterocolitica*, 67–98; *Enterobacter agglomerans*, 83–142; *Serratia marcescens*, 190–241; and *Hafnia alvei*, 200–241. Tompkin et al. (1980) found the addition of 100 mg/kg of SO₂ as sodium metabisulfite to canned pork inoculated with *Clostridium botulinum* spores delayed cell growth. The delay was proportional to the concentration of the bisulfite addition. They also noted that the interaction of sulfites with nitrites caused a lowering of the nitrites available for nitrosamine formation. Reddy and Mandokhot (1987) found that minced goat meat could be preserved up to 11 to 13 days if held at 7°C with 450 mg/L of sulfur dioxide added. The effect was inhibition of growth of the flora. Sensory tests showed no adverse results. The shelf life of ground beef was effectively increased from 1.8 days at 7°C storage with no treatment to 12.6 days at 0°C with the addition of 250 mg/kg of sulfur dioxide. The packaging used was a gas-permeable wrapping that allowed oxidative conditions (von Holy et al., 1988). Adams et al. (1987) found that vacuum packaging and a good oxygen barrier film decreased the

TABLE 5.4
Range of Effective Antimicrobial Concentrations of Sulfurous
Acid against Various Genera of Yeast

Genus	Number of Species	Effective H ₂ SO ₃ (mg/L) ^a
<i>Saccharomyces</i>	13	0.10–20.20
<i>Zygosaccharomyces</i>	2	7.2–8.7
<i>Pichia</i>	1	0.20
<i>Torulopsis</i>	1	0.20
<i>Hansenula</i>	1	0.60
<i>Candida</i>	2	0.40–0.60

^a Rhem and Wittmann (1962).

spoilage in sulfite-treated sausage. This was because of the lack of oxygen delaying yeast growth and the production of sulfite-binding substances. Thus the free sulfite, which inhibited growth, was maintained for a longer period. Sodium sulfite addition in sausage was shown to affect biogenic amines. The concentration of tyramine and putrescine increased in the presence of sulfite, but the level of cadaverine was reduced (Bover-Cid et al., 2001). There was no effect on histamine, phenylethylamine, or tryptamine.

Yeast

The use of sulfur dioxide to deplete the wild yeast in grape juice is a standard practice dating back many years (Cruess, 1912). Rhem and Wittmann (1962) determined the inactivation levels of sulfurous acid for a variety of yeast genera (Table 5.4). Goto (1980) determined viable counts of various wild yeasts in grape juice in the presence of sulfur dioxide and found *Torulopsis* and *Saccharomyces* were the most tolerant, whereas *Kloeckera*, *Pichia*, *Rhodotorula*, and several other genera were very susceptible. Haznedari (1979) tested 30 strains of *S. cerevisiae* that had been characterized as “SO₂ resistant” for tolerance to high concentrations of sulfur dioxide. Six strains could grow well at 1000 mg/L, and five others were able to produce adequate amounts of ethanol. In contrast, Carr and Davies (1971) found sulfur dioxide incorporated into growth medium (pH 3.4) at 25 mg/L was sufficient to kill a culture of *S. cerevisiae* (10⁵ CFU/ml) after 8 hours at 25°C. Dott and Trüper (1978) found “killer yeasts” (those yeasts that when grown in mixed cultures cause the death of other yeasts) were high or medium producers of sulfite and were more resistant to sulfur dioxide.

Molecular sulfur dioxide is the most effective form for inhibiting yeast. The amount of molecular SO₂ (mg/L) in wine can be calculated using free SO₂ mg/L/(1 + 10^{pH - 1.81}). Sudraud and Chauvet (1985) found that to maintain yeast stability, 1.5 and 1.2 mg/L of molecular sulfur dioxide were necessary at the finish of fermentation and during aging, respectively. Ough et al. (1988) found that with yeast acclimatized to sulfur dioxide, between 2.0 and 3.0 mg/L were required. The yeast also showed increased resistance to the fungicide dimethyl dicarbonate. pH is extremely important in the effective use of sulfur dioxide. A 10-fold increase in molecular sulfur dioxide occurs between pH 4.0 and 3.0. Any rule of thumb addition, such as “maintain the free SO₂ at 20 mg/L,” for biological stability can be disastrous. In fact, with the pressure to reduce sulfur dioxide content, its use to prevent the growth of yeast in sweet table wines is seldom ever contemplated.

According to Warth (1985), resistance of yeast to sulfur dioxide ranges from 0.05 M free sulfur dioxide for *Kloeckera apiculata* to 2.8 mM for *Z. bailii* at 25°C under aerobic conditions at pH 3.5 in tryptone yeast extract medium. *Saccharomycodes ludwigii* was nearly as tolerant as *Z. bailii*. Once the inhibition was overcome, the growth rates and cell yield were similar. Delfini (1989)

reported the existence of very sulfite-resistant *S. cerevisiae*, *Z. bailii*, *S. ludwigii*, and *Schizosaccharomyces japonicus* in the re-fermentation of sweet champagnes. Growth was found at the highest concentration of free sulfur dioxide used, and there was no correlation with the number of cells in the initial inoculum. In a cell recycled ethanol fermentation system, Chang et al. (1997) showed that sulfite up to 400 mg/L had no effect on *S. cerevisiae* despite a reduction in the bacterial counts in the system at the same concentration.

Spoilage yeast in dry wines are fairly rare. With modern filtration and sanitation technology, cell numbers are depleted to very low levels at bottling. *Brettanomyces* can contaminate a winery, especially wines in barrels, if proper sulfur dioxide levels are not maintained. In normal sweet table wines, the main spoilage yeast is *Saccharomyces*. With this genus, sulfur dioxide, even at the legal limits, does not always inhibit growth and fermentation. Minarik and Navara (1977) reported finding the spoilage yeast *S. ludwigii* in a low-alcohol wine. This particular species was found to be very resistant to sulfurous acid. *S. ludwigii* is also a noted spoiler in sweet grape juice containing high amounts of sulfur dioxide (Jakob, 1978). Sand (1980) suggested that *Z. bailii* was becoming a problem in juices, soft drinks, and wines because of its tolerance to SO₂, alcohol, and low pH. Spoilage by *Z. bailii* in comminuted orange drink was controlled by 230 mg/L of SO₂ at pH 3.1 but not in the base material, which was at pH 3.7 (Lloyd, 1975).

There have been numerous reports on techniques to minimize the amount of sulfur dioxide used in wines (Aerny, 1986a,b; Gomes and da Silva Babo, 1985; Asvany, 1985; Galassi and Mancini, 1985; Valouyko et al., 1985; Hernandez, 1985). Recommendations include cooling the fruit before crushing, adjusting the pH downward if necessary, using yeast that produces minimum amounts of sulfur dioxide-binding components, settling and racking the juices, reducing the amount of sulfur dioxide before or during fermentation, minimizing the air around the wine, using sulfide salts before fermentation, substituting other preservatives at bottling, and eliminating oxygen from bottled wine. Hydrogen sulfide (H₂S) was used (Ubigli et al., 1982) as an alternative for pretreatment of grape juice before fermentation. The treatment was reported to be successful for juice. Schmitt et al. (1983), however, stated that the use of sulfide salts failed to protect wine and left negative sensory characteristics in the wine. Although other agents may act as antimicrobial agents, none seem to be capable of replacing the antioxidant property of SO₂.

Yeast can form sulfite from sulfate via sulfate permease. Some yeast strains can form rather large amounts of sulfur dioxide during juice fermentation (Table 5.5). Suzzi et al. (1985) tested 1700 *Saccharomyces*. They found a majority of the wines in the test to contain about 10 mg/L of total sulfur dioxide (Table 5.6). This shows that most wines will not be free from sulfite labeling requirements even if no sulfur dioxide is added. Dott et al. (1977) determined the cause for sulfite production to be sulfate permease inhibition by methionine. In high-sulfite-producing yeast, the sulfate permease was not repressed by methionine. Proper selection of the yeast strain to avoid high levels of sulfite is an obvious choice. Very little of the sulfur dioxide formed by the yeast remains in the free state.

Breweries have an interest in sulfur dioxide as an antioxidant. Klimovitz and Kindraka (1989) found that endogenous sulfur dioxide varied with starting specific gravity of the brew and the sulfate content of the water used. Vernerova et al. (1983) showed that increasing the dissolved oxygen in the wort from 1 to 2 to 8 to 9 mg/L caused production of 15% to 30% more sulfur dioxide. Strain differences caused variation in the sulfur dioxide produced from 11 to 26 mg/L. Angelino et al. (1989), investigating the sulfur dioxide content of green beer, found no correlation with dissolved oxygen levels in wort and no correlation with ATP sulfuryase or sulfite reductase. Pearlstein (1988) found that extended aeration decreased sulfite production.

Fruit juices and preserves can be protected from microbial spoilage by sulfur dioxide addition. Generally, because the pH is high, excess amounts are used. Even at low pH (2.6 and 3.4), Sethi and Anand (1984) had to use 692 mg/L in carrot preserves to inhibit *Bacillus cereus*. Ranote and Bains (1982) used 350 mg/L of sulfur dioxide to preserve Kinnow, a late-harvest orange juice. Patel et al. (1985) used an initial 1000 mg/L of sulfur dioxide in the last stages of the manufacture

TABLE 5.5
Formation of Sulfur Dioxide by Various Yeasts during Grape Juice Fermentations

Yeast	Sulfur Dioxide (mg/L)	Reference
<i>Saccharomyces carlsbergensis</i>	12	Minarik (1975)
	150	Eschenbruch and Bonish (1976)
	80, 100, 160, 170, 1300	Heinzel et al. (1976)
<i>S. cerevisiae</i>	22, 35	Minarik (1975)
	20, 52, 100	Eschenbruch and Bonish (1976)
	5, 150	Heinzel et al. (1976)
	0, 3, 4, 5	Delfini et al. (1976)
	18–23	
<i>S. pastorianus</i>	85	Minarik (1975)
<i>S. bayanus</i>	0, 5, 500	Minarik (1975)
	300, 500	Heinzel et al. (1976)
	5.5, 35.5, 44, 56, 76	Delfini et al. (1976)
	17, 18, 20	Poulard and Brelet (1978)
<i>S. uvarum</i>	1.5, 3, 21.5	Delfini et al. (1976)
	14, 18, 21, 40	Poulard and Brelet (1978)
<i>S. chevalieri</i>	0	Minarik (1975)
	18, 20	Poulard and Brelet (1978)
<i>S. acidifaciens</i>	26	Minarik (1975)
<i>S. bailii</i>	27	Delfini et al. (1976)
<i>S. rosei</i>	0	Delfini et al. (1976)
<i>S. italicus</i>	0, 1.5	Delfini et al. (1976)
<i>S. rouxii</i>	0	Delfini et al. (1976)
<i>Schizosaccharomyces pombe</i>	0	Delfini et al. (1976)
<i>Torulopsis stellata</i>	0	Delfini et al. (1976)
<i>Kloeckera apiculata</i>	1.5	Delfini et al. (1976)
	12	Poulard and Brelet (1978)
<i>K. magna</i>	0	Delfini et al. (1976)
<i>K. africana</i>	9	Poulard and Brelet (1978)

TABLE 5.6
Amounts of Sulfur Dioxide Found in Test Wines Fermented by 1700 *Saccharomyces* Yeasts (Suzzi et al., 1985)

Number of Strains	Range of SO ₂ Found in Synthetic Medium (mg/L)	Average Total SO ₂ in Wine (mg/L)
1292	<10	10
354	10–20	17
50	20–30	22
2	30–40	34
2	>40	58

of khoa. Kalra and Chadha (1984) stored mango pulp and Maini et al. (1984) stored mango pulp and tomato pulp in polypropylene or high-density polyethylene pouches using sulfur dioxide as a preservative. The former indicated that 350 mg/L of sulfur dioxide was a better treatment than 100 mg/L. The latter used only 150 mg/L of sulfur dioxide to maintain the quality for up to 2 months for mango pulp.

Molds

A number of molds can infect fruit during processing, storage, and transit to market. *Botrytis* species is probably the most prevalent fungi, but others including *Cladosporium*, *Alternaria*, *Stemphyllium*, *Bysochlamys*, *Penicillium*, *Aspergillus*, *Rhizopus*, and *Uncinula* (powdery mildew) also infect fruit.

Whole-fruit storage success has depended largely on the use of sulfur dioxide. Fishman and Karalidze (1984) dipped mandarin oranges in concentrated sulfur dioxide solutions and then bagged the fruit in large polyethylene bags. The fruit maintained quality for longer than a year. Using only 0.2% and no bagging, Yakobashvili and Georgadze (1984) could achieve only 60 days of stability to mold growth in mandarin oranges. Although this shelf life was superior to other chemical treatments tested, for longer stability they recommended redipping every 40 days and storage at 3°C. Katsaboxakis et al. (1981) preserved citron fruit for longer than 560 days in a 2% sulfur dioxide brine solution. Longan and rambutans, fruits similar to litchi nuts, both have shown improved storage by treatments with sulfur dioxide (Wara-Aswopati et al., 1988; Mohamed et al., 1988). Kuwabara et al. (1984) found good mold growth inhibition using a sulfur dioxide treatment for drying persimmons and no effect on the composition of the treated versus the controls. The SO₂-treated fruit was preferred in sensory tests. Sulfur dioxide was tested to preserve apples by Kaul and Mujol (1982). It controlled *Trichothecium roseum*, appreciably controlled *Glomerella cingulata*, suppressed *Penicillium expansum*, and was partially effective against *Rhizopus stolonifer* and *Monilia taxa*. Sharma (1986) performed an almost identical experiment using pears. Again, sulfur dioxide was effective against *G. cingulata*.

Berries have also been successfully preserved with sulfur dioxide. Demetrashvili (1981) stored mulberries for up to 6 months in a solution combination of tartaric acid (1%) and sulfur dioxide (0.2%). Untreated berries molded within 7 days. Spayd et al. (1984) used SO₂ generators to store raspberries at several temperatures. The visual mold counts were less using the SO₂ generators, but the Howard mold counts were not significantly different for the control versus the treated berries. Significant bleaching of the anthocyanins occurred. They did not recommend the treatment for fresh market fruit.

Sulfur dioxide is used as a fungicide on grapes because of the physical damage resulting from transit, humidity, and temperature. Nelson (1979) reported that grapes held at 2°C with 0.1% sulfur dioxide gassing weekly had only minor mold growth and only moderate bleaching of the fruit. At higher concentrations of sulfur dioxide, mold inhibition was almost complete, but severe fruit discoloration occurred. Slow-release sulfur dioxide generators placed into storage containers appear to be beneficial for short-term (2 months) shipments (Nelson and Ahmedullah, 1976). Marois et al. (1986) found that gassing with sulfur dioxide at 200 mg/L three times per week was superior to 2500 mg/L once a week for table grapes in cold storage. Alvarez and Vargas (1983) preferred direct sulfur dioxide fumigation to generators. Most other publications favor generator treatments. Massignan et al. (1984) preferred in-package generators. Benkhemar et al. (1989) successfully stored Moroccan table grape varieties using SO₂ generators for 3 months. Ballinger et al. (1985), using corrugated shipping containers with polyethylene liners and SO₂ generators, stored *Euveitis* hybrid bunch grapes up to 20 weeks. Mansour et al. (1984) used SO₂ generators to hold Egyptian table grapes for up to 4 weeks at 0°C. *Botrytis cinerea* was inhibited for up to 12 weeks, but *Aspergillus niger* and *Rhizopus* were only inhibited for 2 to 3 weeks at 20°C. At 0°C, the latter were completely inhibited for up to 4 weeks.

Byssochlamys is a mold species that is capable of producing the mycotoxin patulin. The mold can exist in the ascospore form, which is highly resistant to thermal processing. Roland et al. (1984 a,b) studied the best fungicide to use of those legally available. They found sulfur dioxide at 50 mg/L in apple sauce and apple juice was sufficient to kill the mold and was the most efficient. Patulin is also inactivated by sulfur dioxide rather rapidly. Roland and Beuchat (1984) also studied the growth and control of this organism in grape juice, with similar results. This organism is not a normal mold found on grapes. The level of sulfur dioxide normally used for juice processing would preclude its development.

OTHER USES

Any vegetable or fruit, raw, dried, frozen, or canned, that is subject to nonenzymatic or enzymatic browning can benefit by proper treatment with sulfite compounds. Vegetables, such as peas, carrots, beans, cabbage, potatoes, and tomatoes, have more stable color and less deterioration if so treated. Dried fruits held in an atmosphere of sulfur dioxide maintain a more natural appearance. The antioxidant effects of ascorbic acid are enhanced by their combined use with sulfites in foods and pharmaceuticals (Schroeter, 1966). The firmness of whole bananas is increased by dipping them into a 500 mg/L SO₂ solution (Levi et al., 1980). Bolin and Jackson (1985) found that adding an oxygen scavenger to packaged dried apricots or apples greatly increased the effectiveness of the sulfur dioxide.

The modeling of sulfur dioxide uptake in prepeeled potatoes was studied (Rodriguez and Zaritzky, 1986) with regard to shape of potato pieces. Equations were derived for holding times at various SO₂ concentrations, dry matter contents, and velocities of mixing for several different shapes of potatoes. Giannuzzi and Zaritzky (1990) studied refrigerated prepeeled potatoes held in plastic film bags with vacuum packing at three temperatures. Spoilage caused by *Pseudomonas* species and Enterobacteriaceae could be held in the lag phase by 100 mg/L of SO₂.

TOXICOLOGY

The toxicology and safety of sulfur dioxide in its various forms has been the subject of many reviews (Institute of Food Technologists, 1975; National Academy of Sciences, 1978; Papazian, 1996). As early as 1896, articles appeared in scientific journals suggesting the possible toxicity of sulfur dioxide. Studies on the median lethal dose (LD₅₀) of sulfiting agents for various animals were compiled by the Select Committee on GRAS (generally recognized as safe) Substances (1976). In amounts that were less than lethal doses but greater than tolerable levels (higher than 62 mg SO₂ per kg body weight), sulfur dioxide resulted in some physiologic changes in rats, such as polyneuritis, bleached incisors, visceral organ atrophy, bone marrow atrophy, renal tubular casts, stunting of growth, and spectacle eyes (Fitzhugh et al., 1946).

Sulfur dioxide greater than 33 mg/L in air can cause distress or even death when inhaled (Amadur, 1975). In general, the cause of rapid death is pulmonary dysfunction, indicated by pulmonary edema, lung hemorrhage, and visceral congestion. Coughing, lacrimation, and sneezing are outward immediate symptoms. Chronic symptoms (Reid, 1963) are hypertrophy of goblet cells and mucous glands, with excess mucous formation and difficulty in clearing the lungs. Some people are more sensitive than others.

Sulfur dioxide injury to the eye is not an uncommon industrial accident. Anhydrous sulfur dioxide hitting the eye is readily absorbed. Because of its high solubility in fat, it penetrates the cornea and causes deep keratitis and iritis (Grant, 1947).

A summary of a number of test studies reported by the Select Committee on GRAS Substances (1976) indicated that no toxic effects were observed for ingested amounts of less than 30 to 100 mg sulfur dioxide per kilogram body weight per day (depending on the experimental conditions and

species). The variation may in part be the result of differing amounts of thiamine administered in these studies. A joint Food and Agriculture Organization and World Health Organization (Joint FAO/WHO, 1967) report estimated that 35 mg sulfur dioxide per kilogram body weight per day was the “no observed adverse effect level” (NOEL) in the rat. For humans, the joint FAO/WHO Committee (Joint FAO/WHO, 1974) established the acceptable daily intake level at 0.7 mg SO₂ per kilogram body weight per day. The Select Committee on GRAS Substances (1976) concluded that the average per capita consumption of 0.2 mg/kg body weight per day was not a hazard to health.

In a reexamination of the findings of the Select Committee on GRAS Substances (1976), the U.S. Food and Drug Administration (FDA) (1985) agreed with the toxicology findings. Their report stated that no teratogenic effects had been reported and noted that sulfur dioxide had variable effects on mutagenicity to bacteria. No *in vivo* carcinogenic or mutagenic effects could be demonstrated with mice or rats. Itami et al. (1989), using Wistar rats, could not show any teratogenic effects with heavy doses of sulfite but did show evidence of fetal toxicity. It appears from all the published reports that normal humans are reasonably tolerant to sulfur dioxide and, unless damaging doses are given, can recover unaffected.

Until the early 1990s, use of SO₂ was considered GRAS. However, studies indicated that certain asthmatic individuals were at risk by consuming relatively small amounts of sulfites. Mathison et al. (1985) discussed the possible mechanism for sulfite action on asthmatics and concluded that little is completely understood. An extensive review by Gunnison and Jacobsen (1987) discussed in detail the possible mechanisms of sulfite hypersensitivity from which most asthmatics suffer. The review by Nicklas (1989) particularly stressed that many pharmaceuticals are preserved with sulfur dioxide. Linn et al. (1987), in a replicated dose-response study, found that normal nonasthmatics were not affected by doses in the airway of up to 0.6 ppm, but people with asthma responded to this dose level. There are many more papers and reviews on sulfite-induced reactions in people with asthma, but the medical aspects are beyond the scope of this chapter.

The food situation in relation to sulfite reactions is pertinent. In 1983, the FDA (1983) noted that they had received 90 reports of food-caused, sulfite-related, adverse reactions, including one death. Since the monitoring of food reactivity to sulfites started in the 1980s, there have been 1132 consumer complaints describing adverse reactions, and 48.6% of these cases were classified as severe (Warner et al., 2000). Early on, the FDA considered the use of sulfites on fresh fruit and vegetables in restaurant foods a major problem area. Martin et al. (1986) showed that salad fresheners containing sulfites could, when used as recommended, leave a 900 mg/kg residue of sulfite on the product. If the practice is abused, much higher levels would result. Howland and Simon (1989), studying patients known to be sulfite sensitive, challenged them with lettuce treated with commercial, sulfite-containing fresheners. Those with asthma had restriction in the airways and in one case a life-threatening reaction. Dahl et al. (1986) tested red wine-sensitive people with asthma for reactions against amines and sulfites in wine. They found that sulfur dioxide was the more severe causative reagent in all cases. A death related to a red wine that contained 93 mg/L of total sulfur dioxide was reported by Tsevat et al. (1987). The drinker consumed only one glass but was known to have asthma and sulfite hypersensitivity. Gershwin et al. (1985) reported on the effect of wine (white) with and without sulfur dioxide, in controlled amounts, on volunteer asthmatics. They concluded that some asthmatics could be at risk in consuming wine-containing sulfites. In a recent study, Vally and Thompson (2001) exposed 24 patients with asthma and a strong history of wine-induced asthma to a single high-sulfite challenge (300 mg/L). Only four of the 24 patients had a significant reduction in forced expiratory volume. Wines with lower concentrations of sulfites (<150 mg/L) did not elicit any response. Additionally, Vally and Thompson (2001) exposed 12 wine-sensitive and 6 control asthmatics to wine with increasing concentrations of sulfites. No significant differences were found between the groups in any lung function parameter. The researchers suggested that the role of sulfites and/or wine triggering asthmatic responses may be overstated. Taylor et al. (1988) challenged eight people with asthma, previously selected as SO₂ sensitive by sulfite capsule tests, to a number of foods treated with sulfites. They found mixed responses from

the patients to the various foods and drinks. They concluded that not all SO₂-sensitive people with asthma would react to all foods. Steinman and Weinberg (1986) noted that 5% to 10% of children with asthma were SO₂ sensitive and listed foods and beverages to be avoided in South Africa. More recently, pickled onions and salsa were implicated in adverse reactions in patients with asthma (Gastaminza et al., 1995; Nagy et al., 1995).

The body normally metabolizes sulfite to sulfate by the action of sulfite oxidase (EC 1.8.3.1). If this enzyme is less active, then increased levels of thiosulfate can be found in the urine after ingestion of sulfite. Town et al. (1989) tested this idea and could demonstrate the effect on people with asthma compared with normal people using white wine as the challenge. They cautioned that a better test would be direct measurement of sulfite oxidase activity.

AMOUNTS IN FOODS AND REGULATIONS

Sulfites are considered GRAS substances by the FDA when used in amounts that are in accordance with good manufacturing practices. They are allowed in fruit juices and concentrates, dehydrated fruits and vegetables, and wine. The maximum level of sulfur dioxide allowed in wine was set at 350 mg/L by the regulating body for the U.S. alcoholic beverage industry, the Alcohol and Tobacco Tax and Trade Bureau (formerly the Bureau of Alcohol, Tobacco and Firearms) of the Department of the Treasury. Wines with greater than 10 mg/L sulfites must be labeled as containing sulfites. The amount of sulfites used in food products is dictated by good manufacturing practice. In the United States, sulfites are not allowed in meats, foods recognized as a sources of vitamin B₁, or on fruits and vegetables intended to be served or sold raw to consumers or to be presented to consumers as fresh (21CFR 182). The FDA (1986a) rescinded the GRAS status of sulfites on raw fruits or vegetables and declared that sulfite could not be used on these items. The FDA (1986b) also made labeling of any product containing 10 mg/L or more of sulfites mandatory and defined the method of analysis. The amounts of sulfite in foods were limited by the FDA (1988). Sulfite use on fresh potatoes (any potato not canned, frozen, or dehydrated) was banned but later rescinded in a court action (FDA, 1990). New regulations on sulfites in potatoes are still being developed. Table grape sulfite tolerances were set at 10 mg/kg (Environmental Protection Agency, 1989). In the European Union, directives are set for sulfur dioxide [E220], sodium sulfite [E221], sodium bisulfite [E222], sodium metabisulfite [E223], potassium metabisulfite [E224], calcium sulfite [E226], calcium bisulfite [E227], and potassium bisulfite [E228] (Gould, 2000).

In some countries, sulfites may be used to inhibit the growth of microorganisms on fresh meat and meat products (Kidney, 1974). Sulfur dioxide restores a bright color but may give a false impression of freshness. Sulfite or metabisulfite added in sausages is effective in delaying the growth of molds, yeast, and salmonellae during storage at refrigerated or room temperature (Ingram et al., 1956; Banks and Board, 1982).

The amounts of sulfite used in foods and beverages vary greatly between countries. An estimate of the concentrations of sulfites used in foods as an antimicrobial was listed by Gould (2000) (Table 5.7). Levels of sulfites used in wines in the United States were summarized by Ohkubo and Ough (1987). In the survey, 161 white table wines averaged 121 mg/L of total sulfur dioxide. Red table wines were generally much lower. A German report (Wever, 1987) listed the sulfite composition of a number of foods and beverages. A number of the foods were over the German limit, and many did not declare sulfites on the package. He also found that cooking foods frequently lowered the sulfite to below detectable levels. Barnett (1985) reported that levels of sulfites for Australian foods and beverages ranged from 29 mg/L for beer to 3000 mg/Kg for dried fruit. Sulfites were permitted in wines at 350 mg/L. Nordlee et al. (1985) reported on the sulfite levels in 53 different maraschino cherry samples. The total sulfite levels ranged from 10 to 203 mg/L, with a mean of 52 mg/L.

TABLE 5.7
Application of Sulfites in Foods as Antimicrobials and the Concentrations Used (Gould, 2000)

Food	Use Concentration (mg/kg SO ₂) ^a
Beer	10–30
Fresh fruits	100
Fresh vegetables (onion, garlic, horseradish)	50–1000
Fruit juices	10–100
Fruit-based sauces and related products	50–100
Fruit pulps, purees, and fillings	50–500
Jams and jellies	50–100
Nonalcoholic beverages	20–200
Sausage	450
Sugar confectionary	50
Vinegar	50–200
Wine	100–300

^a Sulfites are allowed only in certain foods in different countries, and concentration varies by country.

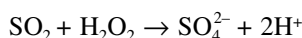
TABLE 5.8
AOAC International Official Methods of Analysis for Sulfites (Warner et al., 2000)

Method Number	Year Adopted	Title	Type of Analysis
892.02	1892	Sulfurous Acid (Free) in Meats	Titrimetry
961.09	1961	Sulfites in Meats	Qualitative Test
962.16	1962	Sulfurous Acid (Total) in Food	Modified Monier-Williams
963.20	1963	Sulfurous Acid (Total) in Dried Fruit	Colorimetry
975.32	1975	Sulfurous Acid in Food	Qualitative Test
980.17	1980	Preservatives in Ground Beef	Colorimetry
987.04	1987	Sulfites (Total) in Foods	Differential Pulse Polarography
990.28a	1990	Sulfites in Foods	Optimized Monier-Williams
990.29	1990	Sulfites (Total) in Foods and Beverages	Flow Injection Analysis
990.30	1990	Sulfites (Free) in Wine	Flow Injection Analysis
990.31	1990	Sulfites in Foods and Beverage	Ion Exclusion Chromatography

ANALYTICAL METHODS

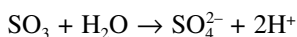
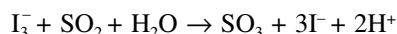
Ough (1988) and Ough and Amerine (1988) gave very detailed reviews of methods for free and total sulfur dioxide determinations in grapes and wines. The official methods of analysis for agricultural and food products are specific in their recommendations for analysis of sulfur dioxide (Table 5.8). Sulfites can be described as free, reversibly bound, or irreversibly bound (Beck et al., 2000). Free sulfites are readily converted to sulfur dioxide upon acidification and can be quantitatively analyzed following distillation. Reversibly bound sulfites are converted to sulfur dioxide only after heat and acid or alkali treatments. Those sulfites that bind food matrices and are not converted to sulfur dioxide with heating or acidic conditions are irreversibly bound. Thiosulfonates are irreversibly bound (Beck et al., 2000). One official method for measuring total sulfurous acid is the modified Monier-Williams procedure. A sample is placed in a distilling flask, acidified, and

heated, and the volatile sulfur dioxide is removed by a stream of nitrogen through a reflux condenser. The reflux condenser must retain all volatile acids except the sulfurous acid. The sulfurous acid is trapped in hydrogen peroxide solution:



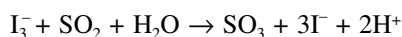
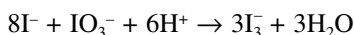
The acid produced by the oxidation of sulfur dioxide to sulfate can be titrated with sodium hydroxide; in addition, the sulfate can be precipitated with barium and measured gravimetrically. This method is good for most products, except dried onions, leeks, and cabbage. Mitsuhashi et al. (1979) showed that sodium sulfide and allyl isothiocyanate gave positive results in the Monier-Williams tests. The optimized Monier-Williams method is used by the FDA to measure sulfites in official samples (Table 5.8) (Warner et al., 2000).

The direct iodine titration method (Ripper) for measurement of total sulfur dioxide is the method used for routine analysis in the wine industry (Ough and Amerine, 1988). Briefly, the sample is made basic to break the bisulfite addition products; it is then acidified, and the freed sulfurous acid is titrated directly with iodine to a starch end point:



This is satisfactory for certain materials, but if other oxidizable substances are present, artificially high results occur. Joslyn and Braverman (1954) reviewed the errors associated with iodine titrations. Vahl and Converse (1980) made a collaborative study of the Ripper method for the Association of Official Analytical Chemists and concluded that the poor precision and large systematic error precluded it for adoption as an official method.

The Ripper method for free sulfur dioxide is given by Ough and Amerine (1988). This is a direct iodine titration of the substance at acid pH. The same errors are associated with this procedure as are associated with the Ripper procedure for total sulfur dioxide. Schneyder and Vlcek (1977) reported that iodate was superior to an iodine standard solution. Excess iodide is added to the sample, and it is then titrated with the iodate solution to a starch end point. The reactions are as follows:



The advantage is a stable standard solution that does not require daily standardization.

The Monier-Williams method can be modified to determine free sulfurous acid. Kielhofer and Aumann (1957), Paul (1958), Burroughs and Sparks (1964b), Rankine and Pocock (1970), and Ough and Amerine (1988) described the method and equipment needed. Basically the sample is sparged with gas for 12 to 15 minutes, and the hydrogen ion produced in the peroxide solution is titrated. Free sulfur dioxide is easily measured in white wines by the Ripper method, but determinations on red wines are more accurately done by this variation of the Monier-Williams method.

Sulfite test strips for protection of people with asthma who are hypersensitive to recognize that the food contained sulfites were questioned by Nordlee et al. (1988) and Wanderer and Solomons (1987). False-negative and false-positive results were found by both groups. Schwedt (1986) considered the test strips satisfactory but noted that if dyes or natural pigments came in contact with the cellulose, false results were recorded.

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6 Nitrite

R. Bruce Tompkin

CONTENTS

Research History	171
Before 1950.....	171
Summary for before 1950.....	174
1950–1960.....	174
Summary for 1950–1960.....	176
1960–1970.....	177
Shelf-Stable Cured Meats	177
Perishable Cured Meats	179
Summary for 1960–1970.....	181
1970–1980.....	182
Shelf-Stable Cured Meat and the Perigo Factor.....	182
Perishable Canned Cured Meat and Slurries of Meat.....	185
Vacuum-Packaged and Fermented Meats	188
Bacon.....	189
Tests with <i>S. aureus</i> and Other Bacteria	191
Summary for 1970–1980.....	192
The Perigo Factor in Cured Meats	192
The Perigo Factor in Media.....	193
Perishable Cured Meats	193
Mechanism of Nitrite Inhibition	193
1980–1990.....	194
Assessment of the Botulinal Risk in Cured Meats	195
Botulinal Challenge Tests	196
Perigo Factor	198
Alternatives to Nitrite for Botulinal Protection.....	199
Mechanism of Nitrite Inhibition	200
Miscellaneous Studies in Laboratory Media	202
Spoilage of Cured Meats	203
Summary for 1980–1990.....	205
Since 1990.....	206
Fermented Meats and Dry Cured Hams.....	207
Alternatives to the Use of Nitrate or Nitrite.....	210
Cheese.....	210
Seafood.....	210
Effect of Nitrite on Enteric Pathogens	212
Effect of Nitrite on Yeasts and Molds	215
Green Discoloration in Cured Meats.....	216

Summary for 1990–2002.....	216
Regulations Governing the Use of Nitrite and Nitrate	217
Assay Method	217
Toxicology.....	218
References	221

This review of the antimicrobial properties of nitrite is one among many. It differs, however, in certain respects from other reviews. By generally arranging the information in chronologic order, insight is provided into why the research was performed, the selection of the experimental procedure, and the information that was available to influence the researchers' conclusions. Other comprehensive reviews are available that provide additional information and other perspectives (Anderton, 1963; Benedict, 1980; Duncan, 1970; Ingram, 1974; Lechowich et al., 1978; Pivnick, 1980; Sofos et al., 1979a; Holley, 1981; Roberts et al., 1991; Skovgaard, 1992). Binkerd and Kolari (1975) reviewed the history of nitrite and nitrate as curing agents for meats. Cervený (1980) described many of the changes in the production and marketing of cured meats in the United States since 1900. The status of nitrate and nitrite in food and water until about 1990, particularly in the United Kingdom and Europe, is discussed in Hill (1991). Extensive updates on the chemistry and impact of nitrite on the color and flavor of cured meats (Pegg and Shahidi, 2000) and the microbiologic, toxicologic, and physiologic effects of nitrate and nitrite (Archer, 2002) have been developed. Reported outbreaks of botulism attributed to meat and poultry products and an assessment of nitrite for botulinal protection have been summarized (Tompkin, 1980). Eklund (1982) reviewed the potential risk of botulism in fishery products and the role of nitrite as a preservative.

The many chemical reactions that occur when nitrite is added to meat offer clues to the reactions that may occur with microbial cells. For this reason, the reader should consider reactions with heme compounds (Fox, 1966; Bard and Townsend, 1971, Roberts et al., 1991; Pegg and Shahidi, 2000) and other components of meat (Cassens et al., 1979). The review by Gray et al. (1981) and Pegg and Shahidi (2000) of the effect of nitrite on the stability of cured meat flavor provides additional possibilities.

The use of nitrite and/or nitrate in foods has been influenced by a variety of factors. Initially, their use was to fulfill the basic need to preserve food. As questions were raised from about 1970 to 2002 over the toxicologic safety of nitrite, considerable effort was directed toward verifying the benefits of nitrite relative to control of certain pathogens and quality attributes. At the same time a considerable amount of research was directed toward identifying the risks associated with the use of nitrite in food. This chapter concentrates on the antimicrobial research. It should be remembered that most of the research during that period was driven by the potential risk to consumers from dietary intake of nitrite and the Delaney clause that declared additives found to be carcinogenic in any animal test could not be used in food in the United States.

With the new millennium a different perspective has evolved. Current regulations for the use of nitrite and nitrate and modern, controlled processing conditions provide the benefits of food preservation with no measurable risk to consumers. It is tempting to delete a significant quantity of information from this third revision of the chapter; however, the information is being retained for its historical value and to provide a foundation for future research when nitrite and nitrate are next called into question.

The discovery in 1969 that nitrosamines can be formed in foods containing nitrite led to a series of events. The time and resources expended to resolve the nitrite issue were enormous and worldwide. At the heart of the issue was whether nitrite would continue to be an approved food additive. If nitrite were not available, then cured meats, as we know them, would disappear from the market. It is a curious thought that if nitrite were disapproved, this chapter would not exist in this book. To better understand this complex issue, the reader is encouraged to read the historical

documentary of Cassens (1990). Cassen's text places the research outlined in this chapter into its proper historical perspective relative to the politics and science of the nitrite issue.

Unlike the other antimicrobial agents discussed in this text, there has been a long, controversial history over whether nitrate and nitrite have antimicrobial properties. Until the early 1940s, nitrite was considered to have no value in meat other than for flavor and color development. Nitrate was believed to provide the antimicrobial effect. During the 1940s and early 1950s, data appeared that began to prove the opposite. By the middle 1950s, research had clearly demonstrated that nitrite has significant antimicrobial properties. By then it was also more firmly believed that nitrous acid derived from nitrite in the acid environment of meat is responsible for microbial inhibition. This concept became widely accepted, and the value of nitrate was relegated to serving merely as a reservoir for the generation of nitrite. By the time the nitrosamine controversy arose in 1969 and the early 1970s, the value of nitrate as a preservative had been so degraded that it was almost unanimously agreed in the United States that nitrate could be omitted from most cured meats without loss of antimicrobial protection or preservation.

Clever as the scientific community has been in more clearly defining the antimicrobial properties of nitrite, it remains to be seen whether wisdom has prevailed with the voluntary abandonment of nitrate as an additive to many meat products in the United States. One of the messages from the 1920s is that nitrate serves as a potential reservoir for conversion to nitrite and thus to nitrous acid. Considering that research continues to reveal new interactions among the chemical constituents of cured meat, has the proper research been conducted and interpreted in an unbiased manner to justify the elimination of nitrate from the majority of cured meats? A primary reason for the controversy over the antimicrobial effect of nitrate and nitrite that prevailed into the 1940s was ignorance of the significance of pH. The list of recognized factors influencing the antimicrobial efficacy of nitrite in cured meats continues to grow 60 years later. It is still difficult to design experiments that yield predictable, reproducible results in all instances. We are still ignorant of all the significant factors that influence the efficacy of nitrite or, for that matter, the value of nitrate.

Research of the 1920s and 1930s focused on using nitrite to cure meat, solving a problem of sour hams, and dealing with perishable canned hams that were being temperature abused in the marketplace. Most of the research on sour hams involved test tube experiments with culture media above pH 7.0, despite meat having a pH closer to 6.0. This basic flaw in experimental design led to results that misguided and hardened opinions in the industry for 20 years.

RESEARCH HISTORY

BEFORE 1950

Tucker (1930) studied the proteolytic activity of a *Clostridium putrificum* isolate from sour ham. Proteolysis was inhibited by 4.0% sodium chloride (salt), 44,000 µg/g of sodium nitrate, or 12,000 µg/g of sodium nitrite in a pork infusion broth buffered to pH 7.6 and held at 37°C. He concluded that, of the three, only salt was used in sufficient concentration in commercial hams to prevent proteolytic activity at 37°C. The same results were obtained with *Clostridium putrefaciens* at 23°C. He suggested that nitrite, not nitrate, is responsible for retarding proteolysis by *C. putrificum*.

Tanner and Evans (1933), using *Clostridium botulinum*, *C. putrificum*, and *Clostridium sporogenes*, found that sodium nitrate up to 22,000 µg/g did not inhibit the 12 strains tested in either pork infusion or egg-meat media. At levels between 22,000 and 44,000 µg/g, irregular inhibition occurred among seven botulin cultures. At 44,000 µg/g, 6 of the 7 cultures were inhibited. The authors concluded that nitrate at concentrations as low as 22,000 µg/g cannot be relied on to inhibit putrefactive anaerobes if other conditions are favorable for growth.

Tanner and Evans (1934) reported that 5900 µg/g of sodium nitrite inhibited 9 of 12 strains of clostridia in nutrient broth and all 12 strains in dextrose broth. Levels of 3100 µg/g in pork infusion and 3900 µg/g in egg-meat, the highest levels tested, were not inhibitory to any of the strains,

although the nitrite was added to the media (pH 7.4) before autoclaving. The authors concluded that reliance cannot be placed on nitrite alone to prevent spoilage by clostridia. From tests with various blends of salt, nitrite, and nitrate in peptic digest broth (pH 7.4), raw pork, and cooked pork, Evans and Tanner (1934) concluded that “the most effective component in curing mixtures is sodium chloride” and “the sodium nitrite present apparently produced no effect on the organisms.” Botulinal toxin was produced in the cooked meat and a few samples of raw meat. Repression of toxin formation in the raw meat was a result of other bacteria that caused a decline in pH to inhibitory levels (pH 4.49 to 5.3).

It is curious that Tanner and Evans (1933), citing earlier work by MacNeal and Kerr, state the following:

[P]otassium nitrate in neutral or alkaline solutions exerted no special restrictive effect on bacterial activity. Under these conditions, it was used as a food. In acid solutions, however, the results were quite different. A marked inhibition was noticed. They said that this effect was incomparably greater than that of salt and was best ascribed to the production of small amounts of nitric acid, and of nitrous acid also, in mixtures containing reducing substances. Potassium nitrate was therefore considered to be particularly effective in restricting acid fermentation of organic substances that are already slightly acid. They further believed that the claim of meat packers that small amounts of nitrate in the pickle produced better preservation of the meat, was borne out by their results. It seemed that nitrate was especially valuable in preventing high degrees of acidity or souring of meat. MacNeal and Kerr stated that the effect of saltpeter was probably due to the oxidizing action of the nitrate ion in the presence of hydrogen ion.

It is surprising that the fundamental importance of pH, so clearly stated and available to researchers at that time, was not pursued.

Perishable (i.e., insufficiently heated to be stable at room temperature) canned, chopped, spiced ham and luncheon meats containing nitrate, nitrite, and sugar swelled in 1 to 90 days during temperature abuse at 37°C. Examining 1000 cans representing all manufacturers at the time, the sole cause of the spoilage was found to be nitrate-reducing *Bacillus* species (Jensen et al., 1934). A medium was developed with nitrate, sugar, and cured meat to detect the gas-producing bacteria in raw materials and the plant environment. Nitrite could not replace nitrate. To obtain gas production in the medium, both nitrate and cured meat had to be present. The reason for this specific combination of ingredients remains unknown. Perhaps the cured meat provides a source of heme, as the more recent results suggest (Jacobs et al., 1964).

The opinions expressed by Jensen et al. in 1934 laid the foundation for a new concept. They stated that if nitrate were omitted from the product, the usual fermentative carbon dioxide swells caused by *Bacillus* species would be prevented and conditions for the growth of clostridia and other anaerobes would prevail. Their bold statements that a mixed nitrate–nitrite cure favors most species of aerobes but a nitrite cure “always inhibits fermentation and aids in putrefaction” were to have long-term effects within the meat industry and federal government.

Brooks et al. (1940) described the contemporary method of making bacon in the United Kingdom. After the slaughtering process, pork sides were held at ambient temperature overnight and then moved into refrigeration for 24 hours to achieve 5.6°C in the meat. The sides were then immersed in a tank and cured using a pickle solution containing potassium nitrate. Several interesting questions were raised. Does nitrate serve any function other than as a precursor for nitrite? Does nitrate appreciably retard or inhibit the growth of putrefactive anaerobes? What is the mechanism behind nitrite depletion, and what factors influence depletion? Would the more rapid chilling process as practiced in North America adversely affect the quality of English-style bacon? Can bacon acceptable to the English trade be produced by the use of rapidly chilled meat and with nitrite in place of nitrate? Not all the questions could be answered, but the research led to the following general conclusions. Satisfactory bacon can be produced with the use of nitrite. The characteristic cured flavor of bacon is primarily the result of the action of nitrite. The conversion

of nitrate to nitrite in commercial bacon curing brines is mainly the result of the growth of micrococci. The presence of nitrate or microbial action during the curing process is not essential for bacon flavor. Rapid chilling of the meat is not detrimental.

Tarr and coworkers published a series of reports on the possible use of nitrite for preservation of fish. Tarr and Sutherland (1940) concluded that "as far as can be ascertained from the literature, little or nothing is known regarding the possible bacteriostatic (or bactericidal) action of nitrites in meat pickles or in cured meats themselves." Their tests with fish muscle showed that nitrite delayed spoilage. Subsequent tests (Tarr, 1941) demonstrated the importance of pH to the efficacy of nitrite. At pH 7.01, little or no inhibition was observed. At pH 5.7 and 6.0, complete or strong microbial inhibition occurred.

The effect of pH was more firmly documented in a series of tests in nutrient broth inoculated with a variety of aerobes. Later tests involving *C. botulinum* and *C. sporogenes* yielded similar results (Tarr, 1942). Investigations on the mode of action led Tarr to conclude that nitrite is not inhibitory by sole virtue of its toxicity toward aerobic respiratory catalysts (Tarr, 1941) and that the mode of action was still unknown (Tarr, 1942).

While Tarr was demonstrating that nitrite has strong antimicrobial properties, Jensen and Hess (1941) advocated the virtues of nitrate and perpetuated the belief that the sole function of nitrite was for cure color development. They stated that the literature shows "unmistakenly that nitrate in the cure exerts a definite inhibitory effect upon bacteria." They postulated the anticlostridial mechanisms to consist of partial conversion of nitrate to hydroxylamine, which inactivates catalase and allows the accumulation of hydrogen peroxide, which destroys anaerobes.

Jensen and Hess stated that nitrite reacts with protein during heating and is destroyed, thus leaving the meat in much the same state as freshly cooked uncured meat. Others have shared this concern (Scott, 1955). Even if nitrite had been uniformly accepted as a critical factor in microbial protection, workers at the time would have had difficulty applying the information to commercial practice. How could reliance be placed on an unstable preservative that disappears during processing and storing? This question is still valid.

The purpose of Jensen and Hess' work in 1941 was to determine the specific value of nitrate as a bacteriostat and as an inhibitor of bacterial spore germination and toxigenicity and its effect on the anaerobic flora of cured meats. Two tests were reported with perishable canned ham. In one, nitrite alone inhibited anaerobic putrefaction during 30 days of abuse at 37.2°C. In the other, fewer cans became putrid in the product with both nitrate and nitrite than in the product with nitrite only. Despite these results, the authors concluded that nitrate is beneficial for preventing the growth of putrefactive anaerobes in abused perishable canned ham. In both tests some cans with nitrate swelled because of the growth of *Bacillus* species, thus serving as a desirable indicator of temperature abuse.

Using the spiced ham medium they reported earlier, Jensen and Hess found that heat (93°C for 4 hours) combined with 5000 µg/g, but not 2000 µg/g, of sodium nitrate, prevented growth of *C. sporogenes* during 4 weeks of incubation at 37.2°C. The authors suggested that the combination of heat, nitrate, nitrite, and salt caused destruction of anaerobic spores at much lower temperatures.

Yesair and Cameron (1942) pursued this idea but concluded that curing salts do not assist in thermal destruction but inhibit outgrowth. Stumbo et al. (1945a) reached the same conclusion from tests in meat processed at 116°C. They also reported (Stumbo et al., 1945b) that nitrite appreciably delayed germination, although salt was the stronger inhibitor. Nitrate, alone or in combination with other ingredients, did not appreciably influence spoilage in tubes processed from $F_0 = 1$ to 16 and held for a year at 28°C.

Jensen et al. (1949) also examined the combined effect of heating and curing salts. Greatly increased inhibition occurred in tubes of pork heated in the range of 50°C to 65°C for 30 minutes. Higher temperatures, longer heating times, or repetitive heating did not increase this effect. Within the levels normally added to canned ham, increasing salt and nitrite caused increased inhibition. However, increasing nitrate did not increase the inhibition of *C. sporogenes* 3679.

Vinton et al. (1947) found the heat resistance of spore crops was not altered by adding nitrite and nitrate to the meat on which the spores were grown, but the condition of the meat was a factor. Spores produced in raw, pasteurized, and sterilized meat were of low, intermediate, and high heat resistance, respectively.

SUMMARY FOR BEFORE 1950

A summary of the state of knowledge by 1950 might include the following:

1. The relative roles of nitrate and nitrite as preservatives in cured meats were unclear. Strong opinions were expressed that nitrite alone was ineffective at the levels used in commercial practice.
2. Nitrate was promoted as an inhibitor of anaerobic spoilage and to enhance the swelling of perishable canned cured meat by *Bacillus* species when temperature abused.
3. Nitrite was clearly shown to be an effective antimicrobial agent, especially if the product pH was below 7.0.
4. The disappearance of nitrite during processing and storage was assumed to make it an unreliable preservative.
5. Salt at the levels used at that time was considered the cornerstone for the preservation of cured meats.
6. The concept of a combined effect of heating in the presence of curing salts was proposed. Although thermal destruction was not shown to be enhanced by the presence of curing salts, there was increased inhibition of outgrowth of surviving spores by salt and, to some degree, by nitrite. Nitrate was without effect.
7. An underlying factor in the research before 1940 was the unfavorable experience of 1928 and 1929 with temperature-abused perishable canned hams. Halvorson (1955) later discussed the widespread problem of temperature abuse at the retail level. It is perhaps for this reason that Jensen believed so strongly about the need for nitrate in perishable canned cured meats. During the 1930s some processors incubated perishable canned cured meat at 37°C as an index of “keeping quality.” This experience may have led to improvements in meat handling and equipment sanitation.

1950–1960

Research of the 1950s clarified the relative significance of salt, nitrate, and nitrite as preservative agents and expands this information to other microorganisms.

Steinke and Foster (1951) investigated the effect of packaging liver sausage in an oxygen-impermeable film (Saran™) that was gaining acceptance in the industry. Salt was found to be the major factor retarding botulinal outgrowth in temperature-abused products. The basic formulation resulted in a product having a moderately high brine of 5.05% to 5.37% and a pH range of 6.1 to 6.5. Adding 200 µg/g of sodium nitrite was considerably more inhibitory to toxin formation than the addition of 1000 µg/g of sodium nitrate; however, a combination of both nitrite and nitrate was the most inhibitory.

Bulman and Ayres (1952) found that levels of salt in excess of 4.4% or sodium nitrate in excess of 40,000 µg/g were required to prevent spoilage from anaerobic spore formers in pork. In the absence of added salt, increased inhibition of PA 3679 was obtained as sodium nitrite was increased from 400 to 800 µg/g. A mixed cure of salt (3.5%) and nitrite (150 to 170 µg/g) was much more effective than either substance alone. Nitrate at the level permitted in meat (1250 µg/g) was without effect, even in the presence of salt or nitrite. A mixed cure of all three (salt, nitrate, and nitrite), however, yielded the maximum inhibition. The question was raised whether the growth of spoilage organisms is influenced by the depletion of nitrite to noninhibitory levels.

Henry et al. (1954) reported that at pH 7.5 or above, nitrite enhanced bacterial growth in curing brine. A pH of 5.6 to 5.8 was optimal for antibacterial efficacy. At pH 5.3 or below, nitrite rapidly disappeared and was ineffective. Nitrite was more inhibitory in the presence of ascorbate.

Castellani and Niven (1955) stated that nitrite was not known to have any practical preservative value against those organisms not inhibited by the high salt content in cured meats. They investigated why *Staphylococcus aureus* is rarely, if ever, found in the interior of cured meats. On adding a filter-sterilized solution of nitrite to a broth medium, less nitrite was necessary for inhibition as the pH was decreased from 6.9 to 5.05. The authors postulated that nitrous acid was responsible for the bacteriostatic action of nitrite. Additional tests showed that if the broth medium (pH 6.55) was autoclaved with glucose, a very small amount of added nitrite prevented staphylococcal growth when incubated anaerobically. This effect was reversed by adding sulfhydryl compounds.

Lechowich et al. (1956) examined whether curing salts plus the anaerobiosis of a vacuum package would inhibit the growth of *S. aureus*, as suggested by Castellani and Niven (1955). Tests in broth media and raw pork led them to conclude that *S. aureus* growth can occur in any combination of salt, nitrate, and nitrite that is palatable and permissible. They found *S. aureus* to die rather rapidly in ham-curing pickle, unless protected by meat juices. Also, *S. aureus* was killed in hams when heated to an internal temperature of 58.3°C, the legal requirement for trichina destruction.

Scott (1955) concluded from the literature that because nitrate exhibited relatively poor antimicrobial inhibition and nitrite, although effective, was unstable, the control of salt concentration and resultant water activity was the most reliable bacteriostatic system for cured meats. As late as 1957, this cautious view of the inhibitory effect of nitrite was expressed by Eddy (1957): "Taken in their totality, these observations leave no doubt inhibition by nitrite is at least a possibility."

An international symposium (Ann. Inst. Pasteur, 1955) on semipreserved meats in hermetically sealed containers included a curious debate. At that time European microbiologists commonly preincubated perishable canned cured meats before microbiological examination. The debated issue was the time and temperature for preenrichment. Today, a similar debate would include the time elapsed since manufacturing.

Halvorson (1955) cited a private communication from L. B. Jensen, who performed a series of tests with spiced ham inoculated with different strains of *C. botulinum*. Jensen concluded that if the brine content were less than 5.0%, the spores could germinate, growth would occur, and toxin would be produced. At 6.0% brine or above, toxin formation was inhibited with a heat treatment as low as $F_0 = 0.27$. Germination was apparently prevented because viable spores could be recovered. Earlier, Dack (1949) reported toxin inhibition in meat with brine levels above 6.4%. In North America, brine values traditionally have been calculated at $\% \text{ brine} = (\% \text{ salt} / \% \text{ salt} + \% \text{ water}) \times 100$. In the United Kingdom, salt levels may be expressed as the percentage brine as just described or as the percentage salt (wt/vol or wt/wt) on the water phase using the equation $\% \text{ brine or } \% \text{ salt} = (\% \text{ salt} / \% \text{ water}) \times 100$.

During the mid-1950s the U.S. Department of Agriculture (USDA) issued a regulation to assure the safety of new perishable canned cured meat products (e.g., 1 1/2 lb canned ham). Because the procedures traditionally used for large canned hams had a history of safety, these existing products were not affected. The requirements imposed on new products included a brine level of 5.5%, the addition of 1.75% sugar, and 1 ounce of sodium nitrate per 100 pounds of meat. This combination of ingredients was derived from the literature and unpublished results from Jensen, who concluded that 1 ounce nitrate was optimal for gas formation by *Bacillus* species in temperature-abused perishable canned luncheon meat.

The USDA concluded that the high brine would retard the growth of *C. botulinum*, and in the presence of nitrate, the growth of *Bacillus* species would occur, causing the cans to swell and forewarn consumers of a potential hazard. A typical formula being used for large canned hams at that time included 0.5% sugar, 1/4 ounce sodium nitrate (156 µg/g), 1/8 ounce sodium nitrite (78 µg/g), and a brine level of 3.5%. The brine requirement of 5.5% would have caused consumer

complaints of excessive saltiness in the new products. It was also believed within the industry that the higher nitrate level would have enhanced can corrosion and product discoloration owing to reduction of nitrate to nitrite by iron at imperfections in the lacquer coating of the cans. This brought nitrate under renewed attack.

Although shelf-stable canned cured luncheon meat had been produced for years, the reasons for stability had not been defined. Silliker et al. (1958) used this class of product to show that nitrate played no role in retarding putrid spoilage. As expected, nitrate actively stimulated spoilage by aerobic spore formers. Brine level (0%, 3.5%, or 5.0%) alone was not responsible for stability. The authors considered the key to stability to be the addition of sodium nitrite (78 µg/g) and heat injury to the small number of indigenous spores. They concluded that the stability of shelf-stable canned cured meat given less than a botulinal cook was the result of the combined effect of nitrite, salt, thermal injury to the spores, and a low indigenous spore level. Because viable spores could be recovered from stable commercial product, the primary effect of nitrite was to prevent germination and/or outgrowth of heat-injured spores. This research and a subsequent review by Silliker (1959) established the future direction for research on nitrite.

Greenberg et al. (1959) subsequently demonstrated botulinal spore outgrowth in perishable canned cured meat having brine levels from 3.0% to 7.125% and abused at 29.4°C to 37.8°C for up to 90 days. Sodium nitrite levels were 22 µg/g or less at the time the meat was inoculated. At 5.14% brine or less, the product was organoleptically unacceptable — as the cans swelled — and toxin assays were performed. A significant observation was that at brine levels of 6.25%, 7.09%, and 7.12%, toxin was produced without concomitant organoleptic spoilage. At 8.95% brine, toxin was not produced. There have been no reported attempts to duplicate this effect of brine at levels approaching total inhibition, although there have been several studies under other conditions in which toxin has been detected without obvious organoleptic spoilage.

SUMMARY FOR 1950–1960

As 1960 approached, the following became increasingly clear:

1. Nitrate, *per se*, had no antimicrobial effect, other than its possible influence on water activity.
2. Nitrate could serve as an electron acceptor, permitting the growth of aerobes, such as micrococci and bacilli. Several new reports of unstable commercially canned cured meats containing nitrate appeared (Hankins et al., 1950; Verhoeven, 1950; Eddy and Ingram, 1956).
3. Although nitrite was recognized as an effective antimicrobial agent, its value as a preservative in perishable cured meats was still in doubt.
4. Nitrite was shown to play a significant role in the stability of shelf-stable canned meat. The system providing stability consisted of the combined effect of nitrite, salt, and thermal injury to the low indigenous level of anaerobic spores. The suspected role of nitrite was to prevent germination or outgrowth of surviving heat-injured spores.
5. Brine content was further shown to influence botulinal outgrowth and toxin formation. At brine levels approaching the inhibitory level, toxin was produced without obvious organoleptic spoilage.
6. *S. aureus* was killed during the heating process given to hams. Anaerobic intolerance to nitrite within the interior of ham, as postulated by test tube experiments, was not confirmed.

1960–1970

After 1960 increased emphasis was directed toward the role of nitrite in the total inhibitory system in cured meats and its effect on thermally injured spores. In the course of this research, the observations of Perigo et al. (1967) opened new possibilities. Research was initiated on irradiation for shelf-stable canned cured meats. The increased use of vacuum packaging raised new questions about the microbiological safety of cured meats. Outbreaks of botulism from temperature-abused vacuum-packed smoked fish during the early 1960s added impetus to this research.

Shank et al. (1962) explored whether nitrite or the oxides of nitrogen were involved in the inhibition of Gram-negative bacteria in cured meats. They concluded that nitrite and nitrate have either neutral or stimulatory effects on bacteria and that nitric oxide has virtually no effect. Nitrous acid was considered primarily responsible for the quantitative as well as qualitative changes that occur in the bacterial flora when meat is cured. It was postulated that nitrous acid reacts either with the cell itself or with various constituents of the medium, making them unavailable for subsequent metabolism.

A requirement of hemin for nitrate reduction and growth stimulation under anaerobic conditions for six strains of staphylococci and one strain of *Bacillus subtilis* was shown by Jacobs et al. (1964). Hemin had no effect on nitrate reduction or growth of *Escherichia coli* or *Bacillus polymyxa*. The authors concluded that when oxygen is absent during the growth of some bacteria, heme synthesis is impaired, even if nitrate is present as an alternative electron acceptor. The impaired synthesis of heme prevents the formation of the required cytochrome systems, and nitrate is not reduced anaerobically. Nitrate and oxygen were considered interchangeable as electron acceptors required for heme synthesis in some bacteria, but not in others.

Shelf-Stable Cured Meats

Riemann (1963) supplemented existing information on the incidence, heat resistance, inhibition, and outgrowth of spores with unpublished data from the Danish Meat Research Institute. He concluded that most of the spores are killed during the thermal processing of shelf-stable canned cured meat. One reason given was that spores indigenous to meat emulsions have lower heat resistance than spores produced in laboratory media. He estimated the thermal process caused a 4-log reduction of the indigenous spores. The surviving spores are subsequently inhibited by the salt and nitrite. If, however, the initial spore load in the product is too high, the larger number of spores that survive the heat process increases the probability that at least one will be able to grow despite the preservative system. Another factor in spore destruction is that a thermal process of $F_0 = 0.4$ to 0.6 at the center of the can would yield 10-fold higher values near the surface of the can.

Gould (1964) observed germination, but at a reduced rate, of spores of *Bacillus* species in the presence of less than $300 \mu\text{g/g}$ of sodium nitrite at pH 6. Immediately after germination, development ceased before lysis or rupture of the spore walls. The inhibited spores did not appear to become fully phase dark. Germination was completely prevented by sodium nitrite levels of $750 \mu\text{g/g}$ or higher at pH 6.0. The toxicity of nitrite was 3 to 5 times greater at pH 6 than at pH 7.

The thermal process used during the early 1960s by seven U.S. producers of shelf-stable canned cured meat was reported to range from $F_0 = 0.1$ to 0.5 , with a median of 0.2 (Greenberg et al., 1965). In a study comparing irradiation with conventional thermal processing, canned chopped ham was inoculated with botulinal spores and given 2 thermal processes. If the surviving spore counts between the enzyme-inactivating (74°C internal temperature) and the shelf-stable process ($F_0 = 0.203$) were compared, a 4- to 5-log reduction occurred. Product inoculated with the lowest inoculum level (173 spores/g) had 0.017 viable cells/g remaining after processing to $F_0 = 0.2$. The presence of $25 \mu\text{g/g}$ of residual nitrite after processing and a relatively low brine level of 3.36% did not prevent outgrowth, spoilage, and toxin formation from this low level of survivors during storage at 30°C to 37.7°C for 6 months.

Spencer (1966) concluded from the literature that 3.5% to 4.0% brine, 75 to 150 $\mu\text{g/g}$ of added sodium nitrite to provide about 20 $\mu\text{g/g}$ after processing to $F_0 = 0.1$ to 0.4, and a low incidence of clostridial spores (less than 1/g) were necessary for the safety and stability of shelf-stable canned cured meats.

The thermal process required to achieve shelf stability of canned hams larger than 1 1/2 pounds caused excessive purge (e.g., 30%) and poor meat texture. Kueck et al. (1965) developed a new process for the production of 3-pound shelf-stable canned hams with a purge of about 17% and acceptable texture and flavor. The process generally consisted of injecting a curing solution and then immersion in a cover pickle for 1 to 3 days as was typical for that time. A 3-pound meat portion was filled into each can, and the product was given a long thermal process at a low temperature (e.g., 2 to 2 1/2 hours in 74°C water) to achieve an internal temperature of at least 71°C. The product was then heated for a short time at a high temperature (e.g., 15 minutes at 113°C to 118°C). This process caused the surface of the meat in the can to reach 96°C to 107°C. The product was then cooled in tap water and placed into refrigerated storage (e.g., 30 days at 6°C to 8.3°C) before release as a shelf-stable product. They demonstrated that increased stability could be achieved by omitting sodium nitrate and sucrose from the formulation. Increased stability also resulted from improving the canning process to reduce or prevent voids. The success of the process was believed to be the result of a combination of factors, including (1) the destruction of vegetative cells by heating to the initial internal temperature of 71°C, (2) partial destruction of spores by the initial long thermal process but at the same time retaining a high level of residual nitrite, (3) destruction of spores on the surface of the meat by the short but high thermal process, and (4) the germination and autolysis of surviving spores during refrigerated storage of the meat because of the adverse conditions for outgrowth (low temperature and high residual nitrite). The refrigerated storage period was necessary to assure stability. This process was used commercially with the approval of the USDA. The process had to be properly controlled to be successful (Kueck, personal communication).

Schack and Taylor (1966) described a procedure to produce shelf-stable canned cured meat with improved quality as a result of reduced thermal process. The process involved a unique sequence of adding the curing ingredients. First, the meat was ground or chopped and then deaerated and backflushed with nitrogen to displace free oxygen. Ascorbic acid was then uniformly distributed throughout the meat. A nitric oxide-generating material, such as sodium nitrite, was then added and mixed into the meat while under a positive pressure (e.g., 1 psig). The meat mixture was then filled into cans, sealed, and then thermally processed. The process was considered to greatly increase the amount of nitric oxide available for use as an antibacterial agent. This process was tested extensively in at least one commercial plant, but the process was not dependable and was abandoned.

Roberts and Ingram (1966) heated spores in water from $F_0 = 0.0015$ to 1.5 before placing them onto culture media containing salt, nitrite, and nitrate. Their results confirmed the concept of spore injury and increased sensitivity to salt and nitrite proposed by Silliker et al. (1958). Curing salts interfered with some stage of germination and development of the surviving heated spores at concentrations that were ineffective with unheated spores. The two *Bacillus* species were rendered more sensitive to salt by lower heat treatments than clostridia. These data reemphasized the importance of spore contamination level for the stability of shelf-stable canned cured meats. Nitrate had no effect beyond that on water activity. The effect of nitrite was pH dependent and led the authors to support the position that undissociated nitrous acid is the inhibitory agent.

Duncan and Foster (1968a) also reported that heated putrefactive anaerobe spores were less tolerant of salt, nitrite, and nitrate than unheated spores. When heated in the presence of these agents, spores tended to be protected by salt and nitrate, but nitrite enhanced heat injury. When heated and cultured in the presence of the agents, salt and sodium nitrate at 0.5% to 1% (5000 to 10,000 $\mu\text{g/g}$) increased the apparent heat resistance of the spores. At 2% to 4%, heat resistance was decreased. Nitrite was strongly inhibitory, especially at pH 6.0, the normal pH value of canned luncheon meat, and appeared to be the chief preservative against putrefactive anaerobic spoilage.

Duncan and Foster (1968b) also reported that nitrite levels up to 600 $\mu\text{g/g}$ at pH 6.0 allowed emergence and elongation of vegetative putrefactive anaerobe cells but blocked cell division. The newly emerged cells then lysed. Even high nitrite levels (i.e., 40,000 $\mu\text{g/g}$) did not prevent germination or swelling of the spores. Salt levels above 6% prevented germination; levels within the range of brine in shelf-stable canned cured meat (i.e., between 3% and 6%) allowed germination and vegetative cell formation. Sodium nitrate affected neither germination nor outgrowth at levels up to 20,000 $\mu\text{g/g}$.

Perigo et al. (1967) offered a new possibility for the stability of shelf-stable canned cured meat. When nitrite was heated in a laboratory medium, an unknown substance was formed that was extremely inhibitory to the growth of vegetative cells of *C. sporogenes*. The authors postulated that the unknown substance may be formed from the nitrite that disappears during heating. Thus, in the case of shelf-stable canned cured meat, the level of residual nitrite remaining after processing may be an inappropriate measurement of the inhibitory capacity of the product. For this reason, they selected a medium that enhanced the disappearance of nitrite during autoclaving. Because formation of the inhibitor required substantial heating, they reasoned the inhibitor might play a complementary role in the stability of shelf-stable but not perishable canned cured meats. The stability of perishable cured meats was assigned to undissociated nitrous acid. Perigo and Roberts (1968) subsequently expanded the observation of Perigo et al. (1967) to 30 different clostridial strains. Roberts and Garcia (1973) later reported 9 of 14 strains of *Bacillus* species and *Streptococcus durans* to be sensitive to the inhibitor, but not *Streptococcus faecium*, *Streptococcus faecalis*, *Streptococcus faecalis* var. *zymogenes*, or *Salmonella* Typhimurium.

The first attempt to demonstrate the presence of a Perigo-type factor (PTF) in cured meat was unsuccessful (Johnston et al., 1969). Johnston et al. concluded that the inhibitor produced in media is of little or no consequence for explaining the inhibitory effect of nitrite in commercially produced canned cured meat.

The interrelation of thermal process, salt, nitrite, and spore level in shelf-stable canned cured meat was further substantiated by Pivnick et al. (1969). Ground pork inoculated with 1 botulinal spore/g and processed to $F_0 = 0.6$ became toxic in the absence of salt and nitrite. Meat inoculated with 10^6 spores/g remained nontoxic after the same process if sufficient salt and nitrite were present. This required either a brine level of 6.1% alone, one of 4.6% with 300 $\mu\text{g/g}$ of sodium nitrite, or intermediate levels of brine and nitrite. The significance of spore level on the thermal process required for preventing botulinal outgrowth was again demonstrated. Viable botulinal cells were recovered in stable inoculated product 18 months after processing. The authors concluded that the safety of some shelf-stable cured meats is the result of the scarcity of botulinal spores in the raw materials. The questions were raised as to what constitutes a safe process and at what point are the products underprocessed. Despite the research and experience since the commercialization of these products, to date there has been no agreement on the lower limits of brine, nitrite, and the thermal process required for the safety of shelf-stable cured meats given less than a botulinal cooking.

Four possible mechanisms by which nitrite may inhibit heat-injured spores in shelf-stable canned cured meats were investigated by Labbe and Duncan (1970). Of the four, their results supported only one: the inhibition of outgrowth of heat-injured spores. When testing the PTF formed with nitrite levels up to 10 times greater than those commercially accepted, the inhibitor did not prevent germination of most of the intact or heat-injured *C. perfringens* spores.

Perishable Cured Meats

Thatcher et al. (1962) questioned the safety of vacuum packaging. Growth levels of *S. aureus* were similar in back bacon packaged under nitrogen, vacuum, air, and 5% carbon dioxide in oxygen, and enterotoxin was reportedly produced under all four packaging conditions. The bacon exposed to air or carbon dioxide in oxygen was obviously spoiled. Because the bacon incubated under

nitrogen or vacuum was organoleptically acceptable, the authors recommended that vacuum-packaged nonsterile processed foods be stored in the frozen state.

Gough and Alford (1965) showed that spores of *C. perfringens* inoculated into raw hams could survive the thermal process, but no attempt was made to determine whether they could multiply if the hams were subsequently temperature abused. From studies in thioglycollate medium containing salt, nitrate, and nitrite, the authors concluded that the levels used in ham would permit growth. No mention was made of pH and none of the agents were tested in combination.

Brownlie (1966) demonstrated the combined inhibitory effect of sodium nitrite concentration, pH, and temperature on a *Microbacterium* species in all-purpose Tween (APT) broth. At pH 7.0, the presence of nitrite caused very little or no inhibition. At pH 6.0 and below, increasing the amount of nitrite from 25 to 200 µg/g caused progressively greater inhibition. Nitrite was more inhibitory at 0°C than at the other temperatures tested (10°C and 25°C). Another 25 strains of microbacteria failed to grow at pH 5.5 in the presence of 200 µg/g of sodium nitrite. Of 25 lactobacilli from cured meat, 21 were able to grow under the same conditions. This would help to explain the dominance of the lactobacilli as spoilage organisms in many perishable cured meats.

Several studies on the growth of *C. botulinum* in vacuum-packaged perishable cured meats were reported during this period. Schmidt and Segner (1964) observed increased delay in the growth of four type E strains in culture media (neutral pH) as the nitrite level increased from 0 to 200 µg/g. A similar effect occurred with increasing salt from 0% to 4% during incubation at 7.8°C or 10°C, but not at 29.4°C. This suggested that salt becomes more inhibitory as storage temperatures are decreased. The four strains failed to produce toxin during 5 weeks at 10°C in sliced bologna (2.7% brine, pH 6.1, 34 µg/g of residual nitrite). Schmidt and Segner (1964) concluded that factors or a combination of factors provided a much longer shelf life than might be expected from salt content alone.

Christiansen and Foster (1965) found type A botulinal toxin production to occur at the same rate on bologna whether packaged with vacuum or without. The time required for toxin production decreased as the abuse temperature was increased (22°C, 30°C, and 37°C). When *S. aureus* was inoculated onto sliced chopped ham, lower maximum population levels occurred in vacuum packages than in nonvacuum packages at 15°C and 22°C.

Pivnick and Bird (1965) found the oxygen permeability of packaging films to influence spoilage but not toxigenesis of sliced cured meats inoculated with *C. botulinum*. Their findings that the type of cured meat influenced toxin production were particularly significant. Bologna was relatively resistant to type A toxin production but not ham. Cooked sliced ham from five producers inoculated with type E spores yielded variable results at 30°C, which was thought to be the result of product differences in brine and residual nitrite levels. Toxin was not produced at 10°C or lower. On sliced bologna, type E toxin was not produced, even at 30°C. The combination of brine (3.75%), residual nitrite, a fermentable carbohydrate, and decreasing pH during temperature abuse were the suggested inhibitory factors. Similar results were obtained with jellied corned beef stored at 15°C and 25°C. Within 1 week the product pH had decreased from 6.0 to 4.6. In jellied ox tongue, type E toxin was produced at 10°C to 25°C but not at 5°C. A total of 90% of the samples held at 25°C remained above pH 6.0. Failure to obtain toxin production at 5°C may have been influenced by pH because 13 of 15 samples had a pH of 5.7 or less after 4 weeks.

The significance of brine level and storage temperature on botulinal toxin formation (types A and B) in vacuum-packaged meats was studied by Pivnick and Barnett (1965). Toxin was produced within 7 days at 30°C in bologna (3.8% brine), but not at 25°C if the brine was 5.1% or higher. A decrease in pH likely occurred during storage and prevented toxin formation in the other variables. In sliced ham, toxin was produced at 20°C to 30°C but not at 15°C. The rate of toxin formation decreased as the brine level increased from 3.1% to 4.8%. At the highest brine level (4.8%), toxin was produced only at 30°C. In jellied pork tongue, toxin was detected in 2 days at 2.35% and 3.5% brine and in 4 days at 4.4% brine at 30°C. The effect of brine on type E toxin production was more

dramatic. In jellied pork tongue, toxin was detected after 4 days with 2.35% and after 8 days with 3.5% brine at 20°C. Toxin was not produced in product with 4.4% brine. All three products (bologna, ham, and jellied pork tongue) were formulated with commercial levels of nitrate and nitrite. Although not mentioned, as the brine level increased in the bologna and ham, an increase in the level of residual nitrite also occurred.

The effect of nitrite levels on types A and B botulinal toxin production in vacuum-packaged sliced ham (4.1% brine) and jellied pork tongue (3.8% to 4.3% brine) was studied next (Pivnick et al., 1967). In both products a marked delay in toxin production occurred at 20°C. At each temperature (20°C, 25°C, and 30°C), increasing the nitrite level increased the time for toxin production. The authors concluded that heat injury of spores is not necessary for nitrite to prevent toxin production in cured meats.

Warnecke et al. (1967) reported type E botulinal toxin production in ground beef held at 22.2°C for 3 to 18 days. Toxin was also detected in bacon held at 12.8°C and 22.2°C, but this is highly questionable, considering the bacon had a brine level of 5.28%. Toxin was not produced in either bologna or country cured ham.

Blanche-Koelensmid and van Rhee (1968) described a smoke ring sausage produced by stuffing the sausage mixture into casings and smoking at 25°C to 35°C, during which time naturally occurring lactics drop the pH to 5.5 to 5.8. The raw product was then sold at ambient temperature for cooking at home. The product had been changed to a shelf-stable fermented product precooked to 80°C in an oxygen-impermeable casing. Using juices expressed from the newer sausage product, they determined that type B botulinal outgrowth was inhibited during 4 weeks at 30°C if the juice had a combined pH of 5.8 or less, a brine level of 4.5% or above, and an input nitrite level of 500 µg/g or more.

An additional factor influencing the apparent significance of nitrite is the growth of competitive flora. Kafel and Ayres (1969) demonstrated that the growth of enterococci in culture media and, more importantly, in perishable canned hams can inhibit the growth of *C. botulinum*, *C. perfringens*, *C. sporogenes*, and *Lactobacillus viridescens*.

Riemann (Anon., 1968) emphasized maintaining adequate salt levels to assure the microbiological safety of perishable vacuum-packaged cured meats. He concluded that *C. botulinum* type A seemed to be completely inhibited by 4.5% brine at pH 5.3, 5.5% brine at pH 6.1, and 8.6% brine at pH 6.5. Type E botulinal growth is inhibited by 2% brine at pH 5.2 and 3% brine at all pH values. The growth of *S. aureus* is inhibited by more than 4% brine at pH 5.1, 9% brine at pH 5.3, and more than 10% brine at pH 6.9.

SUMMARY FOR 1960–1970

Progress made during the 1960s toward defining the role of nitrite in cured meats included the following:

1. Evidence was presented that the antimicrobial effect of nitrite is related to the formation of nitrous acid.
2. The system responsible for the shelf stability of canned cured meats given less than a botulinal cooking was studied by several research groups. All of them reached the same general conclusions as those set forth by Silliker et al. (1958).
3. The concept of thermal injury to spores that survive the processing of shelf-stable canned cured meats was confirmed.
4. The process of outgrowth was more sensitive than spore germination to nitrite.
5. A new explanation for the shelf stability of canned cured meat was proposed. Perigo et al. (1967) suggested an inhibitory substance was formed from nitrite, which disappeared during the thermal process.

6. An early test on the effect of vacuum packaging on the safety of perishable cured meat led to the recommendation that they be frozen to prevent the development of microbial health hazards.
7. Certain cured meats are more prone to supporting botulinal growth. Factors influencing growth included brine level, pH, residual nitrite, storage temperature, and inoculum level. Limited data indicated the lowest temperature reported for growth of type E on cured meats to be 10°C. Lücke et al. (1980) would later report growth at 8°C in liver sausage.
8. The significance of decreasing pH on botulinal inhibition in cooked cured meats with sufficient fermentable carbohydrate was reported for the first time.
9. The growth of enterococci in perishable canned hams was reported to inhibit several clostridial species.

1970–1980

From the outset of the 1970s, the formation of nitrosamines in cured meats was an issue of immediate concern. Throughout the decade pressure increased to reduce or eliminate nitrate and/or nitrite from cured meat. The timely report of Perigo et al. in 1967 opened the possibility of a breakthrough on the mechanism of nitrite inhibition. Considerable effort was applied toward isolating and characterizing the Perigo factor, with the goals of greater understanding and its possible use as a preservative to replace nitrite.

Research during this period can be grouped into five categories by the approaches used. They include tests in (1) test tubes or Petri plates using controlled media and conditions, (2) water slurries of meat processed in bottles, (3) meat products processed in tubes or cans, (4) meat products produced and packaged in pilot plants, and (5) commercially produced meats. Each of the approaches had its favorable and unfavorable features. As in the past, the test tube approach was productive, but not all the results were directly applicable to commercially cured products. The complex, dynamic system in which nitrite plays a role in cured meats has been virtually impossible to duplicate in microbiological media.

Shelf-Stable Cured Meat and the Perigo Factor

Pivnick et al. (1970) used an aqueous meat mixture without salt or other additives to investigate four possible roles of nitrite in shelf-stable canned cured meats: (1) nitrite induces germination during the heating process, and the germinated spores are killed by heat; (2) nitrite potentiates direct killing by heat without the need for prior germination; (3) nitrite increases the germination rate of spores that survive heating; and (4) these rapidly germinating spores are then inhibited by the nitrite remaining after processing and subsequently die before the nitrite has decreased appreciably. Of the four possible roles, nitrite at the level used commercially (200 µg/g) did not enhance destruction during heating or germination during subsequent storage. The chief value of nitrite appeared to be its ability to prevent growth from spores that survive and germinate following processing.

Ingram and Roberts (1971) heated botulinal spores in water and in water with salt (4%) and sodium nitrite (200 µg/ml) before subculturing into media with and without salt (2%) and sodium nitrite (50 µg/ml). Salt and nitrite had little effect on the degree of thermal destruction, but their presence in the culture medium markedly reduced the recovery of the heated spores. The data were discussed in terms of applying the D concept to shelf-stable canned cured meat.

Matsuda and Sekiguchi (1971) studied the combined effect of heat (105°C, 110°C, and 120°C) and curing salts on the destruction of *Clostridium subterminale* spores. Neither sodium chloride (3%) nor sodium nitrate (0.1%) affected thermal destruction when tested alone. Sodium nitrite alone caused increased destruction. A combination of 3.0% salt, 0.1% sodium nitrate, and 0.01%

sodium nitrite caused the greatest destruction when heated. Small differences in the amount of sodium nitrite below 0.01% strongly influenced the results, but not at levels above 0.01%.

The effect of heating spores of PA 3679 at 115.5°C in ground pork containing 2.8% salt, 200 µg/g sodium nitrite, and 0.5% sodium tripolyphosphate was studied by Nordin et al. (1975). Omitting any one or all three of the additives did not affect thermal destruction, thereby confirming earlier research, as already discussed. The effect of nitrite, salt, pH, and heating at 115.5°C on the outgrowth of surviving spores was also examined by placing the heated tubes at 23°C for up to 1 year. The major factor influencing outgrowth was pH; nitrite and salt levels had lesser effects, although they were "clear and undisputable subsidiary effects." The effect of all three factors (pH, nitrite, and salt) appeared to be additive. Outgrowth was determined by blackening of the tubes as a result of ferric citrate having been added to the meat. Because it would later be found that iron concentration can influence the antibotulinal efficacy of nitrite in perishable canned cured meat given a milder heating, did the addition of ferric citrate influence the results? The interaction of iron and nitrite has not been investigated in shelf-stable canned cured meats heated in steam under pressure (e.g., 115°C), as opposed to perishable canned cured meats heated in hot water to a minimum internal temperature of 66°C. Preliminary data, however, exist (Christiansen et al., personal communication), showing a similar iron–nitrite interaction with *C. sporogenes* strain PA 69-82 in perishable canned cured meat as occurs with *C. botulinum*. A similar response with strain 3679 would not be unexpected.

Farkas et al. (1973) studied the effect of adding nitrite to the brine solution for canned Vienna sausages. The sausages were prepared with nitrite-containing curing salt, cooked, and then packed into cans. One of the sausages in each can was inoculated with *C. sporogenes* spores to provide a level of 10^4 spores per can. The cans were then filled with brine (3% salt), sealed, and heated to varying degrees. Sausages packed in brine with 400 µg/g of sodium nitrite and processed to $F_0 = 0.35$ were as stable as sausages in brine without nitrite and processed to $F_0 = 2.6$. A 1:1 extract was next prepared with 3% saline and ground, cooked sausages, the solids removed, the liquid placed into tubes, inoculated (10^5 /ml), overlaid with paraffin oil, and heated ($F_0 = 0.4$). The extract had 2.6% salt, a_w 0.966, and pH 6.4. Between 120 and 200 µg/g sodium nitrite provided stability through 5 weeks of storage. A significant observation was that residual nitrite did not undergo normal depletion in the extract. After a loss of 15% to 25% during processing and an initial decline, residual nitrite levels remained above 125 µg/g throughout 80 days at 30°C.

Johnston and Loynes (1971) obtained Perigo factor formation in the Perigo et al. (1967) medium and reinforced clostridial medium but not in liver veal or Wynne fluid media. This emphasized the importance of medium selection when studying the antimicrobial activity of nitrite and relating the results to commercially produced meats. Aqueous meat suspensions prepared from raw, normal, and overheated (115°C for 90 minutes) canned luncheon meat required 500, 150, and 50 µg/g of sodium nitrite, respectively, for inhibition. The residual nitrite values of the four media and three meat suspensions were about the same after preparation. Considerable differences were noted in their redox potentials. Reducing agents (e.g., ascorbate and cysteine) enhanced the antibotulinal effect of the meat suspensions but did not induce Perigo factor formation.

Ashworth and Spencer (1972) described a model system of ground pork (25 g) in 1-ounce bottles. The pork was autoclaved before or after adding nitrite, held overnight for nitrite equilibration, and then inoculated with vegetative clostridial cells. The inhibitory level of nitrite was not influenced by pH within the range 5.7 to 6.3. Reducing agents (e.g., sodium ascorbate; cysteine; and, to some degree, thioglycollate) enhanced the effect of nitrite. It was reported that less nitrite was required for inhibition when it was added before autoclaving. These observations led to the conclusion that a Perigo-type effect was detected in the meat system. The magnitude of the effect was much less than was previously reported in culture media. Perigo et al. (1967) reported equivalent inhibition with 8 µg/g of nitrite added before heating the medium and 52 µg/g added after heating the medium. In ground pork, Ashworth and Spencer (1972) reported the equivalent levels to be

60 to 70 and 90 to 100 $\mu\text{g/g}$. Perigo et al. (1967) based their conclusions on the added level of nitrite; Ashworth and Spencer based their conclusions on the residual level of nitrite. Actually, more added nitrite was required in the pork if the nitrite was added before (300 $\mu\text{g/g}$), rather than after (150 $\mu\text{g/g}$), autoclaving.

It was originally suggested (Perigo et al., 1967) that the nitrite that disappears during heating may be involved in the formation of the unknown inhibitor. Ashworth and Spencer (1972) found no relation between the amount of nitrite lost during heating and the inhibition obtained. With excessive heating, higher levels of added nitrite were required for inhibition. The additional nitrite provided comparable residual levels after the various heat treatments, which indicates that residual nitrite influenced the results.

When spores were used, results similar to those just mentioned were obtained during the early stages of temperature abuse. However, with prolonged storage (42 days at 37°C), higher initial residual nitrite levels were required for inhibition. In summary, the conclusion that a Perigo effect was demonstrated must be tempered with the observation that, on the basis of added nitrite, more was required when added before heating the meat. If residual nitrite is a valid means for comparison, the Perigo effect was of low magnitude. Residual nitrite, *per se*, strongly influenced clostridial inhibition. The data obtained with the spore inoculum should be more relevant to shelf-stable canned cured meats.

Ashworth et al. (1973) extended this research to the milder heating given to large perishable canned hams and obtained similar results. Adding salt (3.5% brine) to the meat had no effect on the inhibitory levels of residual nitrite but appeared to reduce the level of added nitrite required for inhibition. In this case alone, the levels of nitrite required to obtain inhibition were about the same or lower if added before heating than if added after heating. The inhibitory levels of residual nitrite for two heating processes — heating from 80°C for 4 hours and heating from 20°C to 70°C over a 4-hour period — did not differ. Similar residual nitrite levels were required to inhibit vegetative cells (160,000/g) added after heating and spores (20/g) added before or after heating. In all cases, less residual nitrite was required for inhibition when nitrite was added before heating. Inhibition by nitrite diminished gradually over a period of 8 weeks at 37°C. Ashworth et al. concluded that a PTF was produced, and it may also play a role in the safety and stability of pasteurized cured meats, such as perishable canned ham.

Johnston et al. (1969) found the inhibitor of Perigo et al. (1967) to be inactivated when added to meat. Pivnick and Chang (1974) considered, however, that although the Perigo inhibitor may not be present in meat, another inhibitory factor may be formed when nitrite is heated with meat. The term Perigo-type factor (or PTF) was selected to describe the effect observed in their test. Shelf-stable canned cured luncheon meat was prepared with 0 to 300 $\mu\text{g/g}$ of sodium nitrite and processed to $F_0 = 0.4$. The product was held until the highest level of nitrite declined to less than 2 $\mu\text{g/g}$. The meat was then inoculated with botulinal spores, which had survived heating to $F_0 = 0.4$ in raw meat juice with 4.5% salt and 150 $\mu\text{g/g}$ of sodium nitrite. The authors found that as the original input nitrite level increased, outgrowth and toxin production decreased. This was considered evidence for an inhibitory factor other than residual nitrite. The amount of PTF formed was related to the amount of original nitrite added to the meat before heating. They concluded that although a PTF was formed, the relative inhibitory effect was quantitatively small. The equation of Pivnick and Petrasovits (1973) was used to estimate the inhibitory effect of the PTF relative to the protection provided by other factors (e.g., thermal destruction of spores and inhibition of the survivors by the salt).

Chang and Akhtar (1974) prepared buffered saline suspensions of canned luncheon meat made with 0, 100, 150, and 200 $\mu\text{g/g}$ of sodium nitrite and processed to $F_0 = 0.4$. The nitrite level of the suspensions was adjusted after heating to 53.5 or 22.1 $\mu\text{g/g}$, deaerated in boiling water, and inoculated. The levels selected (53.5 and 22.1 $\mu\text{g/g}$) were the residual nitrite levels after 0 and 2 weeks in product made with 200 $\mu\text{g/g}$ of nitrite. The depletion rate of the nitrite added to the suspensions was not influenced by the amount of nitrite originally added to the meat. Botulinal

inhibition was related to the original amount of nitrite. A second test using canned product held for 4 weeks at 35°C before preparing suspensions with 22.1 µg/g of sodium nitrite yielded similar results. Decreasing inhibition occurred as the product was held for 0, 2, and 4 weeks before preparing the suspensions. The degree of inhibition was correlated with the amount of nitrite originally added to the product. This correlation agreed with results obtained when canned product was held at 35°C to allow the depletion of residual nitrite and then challenged directly (Chang et al., 1974). It was concluded that a PTF is formed during the heating of canned luncheon meat and that the inhibitor is not stable and loses its activity during storage. Thus, age of product must be considered when investigating the presence or activity of PTF in commercial product or model meat systems.

Roberts and Smart (1974) found that the inhibitor in the medium of Perigo et al. (1967) had a comparable effect on the inhibition of clostridial spores, regardless of the inoculum level or whether the spores were untreated, heat injured, or irradiation injured. There was no evidence of germination (i.e., vegetative cells) in the media with inhibitory levels of nitrite, although the spores remained viable during prolonged incubation. Inoculation of the medium after heating with nitrite and aging for 3 months showed that it was less inhibitory, indicating the Perigo factor is not stable.

Additional studies were reported by 1980 that dealt with the Perigo factor (Mirna and Hofmann, 1969; Wasserman and Huhtanen, 1972; van Roon, 1974; Ashworth et al., 1974; Incze et al., 1974; Mirna and Coretti, 1974; Moran et al., 1975; Hansen and Levin, 1975; Huhtanen and Wasserman, 1975; Asan and Solberg, 1976; Lee et al., 1978; van Roon, 1979a,b). Van Roon (1979a, b) concluded that Perigo-type inhibitors do not contribute to clostridial inhibition in heated cured meats. Debatable as the formation of a Perigo-type in meat may be, common agreement that nitrite, iron, and sulfhydryl compounds are involved in the formation of the Perigo factor in broth media may be significant.

Riha and Solberg (1975a) studied the formation and effect of a PTF on seven strains of *C. perfringens* in a chemically defined medium. They subsequently concluded (Riha and Solberg, 1975b) that inhibition was apparently at the cellular level. They hypothesized that nitrite, in some form, causes inhibition by reacting with enzymes containing functional sulfhydryl groups. Additional data by O'Leary and Solberg (1976) suggested that inhibition of *C. perfringens* may involve an interaction of nitrite, as nitrous acid, with sulfhydryl-containing constituents of the bacterial cell.

Perishable Canned Cured Meat and Slurries of Meat

In addition to research on shelf-stable canned cured meats and the composition and role of the Perigo factor in commercial products, considerable effort was devoted to perishable cured meats. Research on perishable canned cured meat or meat slurries is discussed first.

Tjaberg and Kvale (1972) studied the antibotulinal effect of nitrite (0 to 480 µg/g) added to canned raw meat, shelf-stable canned meat, and perishable canned meat and held at 4°C, 22°C, and 37°C, respectively. At 4°C, predictably, toxin was not detected. At 22°C, the rate of swelling ranged from 11 to 48 days across all variables. Neither swelling nor toxin occurred in some treatments having 240 or 480 µg/g of nitrite. At 37°C, swelling and toxin production ranged from 3 to 17 days, and fewer treatments proved stable. The authors concluded that it was not possible to demonstrate any preservative action of nitrite at the levels (i.e., 200 µg/g) usually used in meat products.

Perishable canned cured pork was studied by Christiansen et al. (1973). Botulinal outgrowth in product abused at 27°C was influenced by the spore inoculum level and the level of nitrite. Nitrate had a slight but statistically significant inhibitory effect that may have been caused by nitrite generated from nitrate. Nontoxic spoilage occurred at 7°C in uninoculated product made without nitrite. It was stated that botulinal inhibition was related to the level of nitrite added during formulation rather than residual nitrite. This was later reassessed by Christiansen (1980), who concluded that residual nitrite is a significant factor influencing botulinal inhibition.

Baird-Parker and Baillie (1974) studied the growth of a large, diverse collection of botulinal strains in a broth medium and a cooked pork medium. Sodium nitrite and L-ascorbic acid were added as filter-sterilized solutions. The number of strains showing growth in broth was found to decrease with increasing nitrite (50, 100, 150, 200 $\mu\text{g/g}$), decreasing temperature (25°C, 20°C, 15°C), decreasing pH (7.0, 6.5, 6.0, 5.5), increasing salt (1.5%, 3.0%, 4.5%, 6.0% w/v), and decreasing inoculum level (10^6 , 10^3 , 10^1). Adding L-ascorbic acid (1.0%) markedly increased the effectiveness of nitrite with none of the 38 strains tested showing growth in broth containing 50 $\mu\text{g/g}$ sodium nitrite. Although less effective, a lower concentration of L-ascorbic acid (0.1%) also increased the efficacy of nitrite in broth but had no effect, pro or con, in the cooked pork medium. Furthermore, a preliminary test in bacon led to the conclusion that L-ascorbic acid does not alter the antibotulinal properties of nitrite in bacon.

Grever (1974) examined the stability of emulsions normally used for preparing cooked sausage and liver sausage. The emulsions were filled into cans and heated. Stability was influenced by nitrite content, level of clostridial spores, product pH, and whether the products were treated by a perishable or shelf-stable thermal process. It was suggested that *Bacillus* species are less sensitive to nitrite than clostridia.

Jarvis et al. (1976) found that heating botulinal spores in an aqueous extract of lean pork at 85°C, 90°C, or 95°C decreased the number that could grow in a recovery medium containing 2.5% salt. Spores heated at lower temperatures (70°C or 80°C) were inhibited by 4.5% but not 3.5% salt. A statistically significant salt–nitrite interaction was observed after heating (70°C to 95°C) only if the recovery medium contained salt levels of 4.5% or above. Spores heated at either 70°C or 95°C were more sensitive to nitrite that was autoclaved in a meat slurry or the Perigo medium than to nitrite added after autoclaving. The spores heated at 70°C and 95°C were equally sensitive to the autoclaved nitrite. Autoclaving nitrite in the Perigo medium resulted in significantly more inhibition than autoclaving the nitrite in the meat slurry; 20 $\mu\text{g/g}$ of nitrite in the Perigo medium and 210 $\mu\text{g/g}$ in the slurry were equally inhibitory. Jarvis et al. recommended that large-scale tests be used to quantify the many interacting factors influencing the safety of perishable cured meats.

This led to the pork slurry system of Rhodes and Jarvis (1976) and used by Roberts et al. (1976) to study the interactions of temperature of abuse, salt, nitrite, and inoculum level on botulinal growth and toxin production at pH 6.0. Temperature of abuse was the most significant factor influencing the rate of botulinal growth. At 15°C, the greatest inhibition occurred with 1.8% or 3.5% salt (on water phase). At 17.5°C, less inhibition occurred. At 20°C, 22.5°C, or 25°C, neither salt level provided much inhibition, even with 300 $\mu\text{g/g}$ of sodium nitrite. At the lower temperatures, a pronounced salt–nitrite interaction occurred. At 20°C, the impact of increasing the nitrite level from 40 to 300 $\mu\text{g/g}$ was minor and became less so as the temperature of abuse increased to 22.5°C and 25°C.

The effect of nitrite in perishable canned cured meat was also reported by Tompkin et al. (1977). The rate at which inoculated cans swelled at 27°C was inversely related to the level of nitrite added to the product. The antibotulinal effect of nitrite was considerably greater than that reported for the meat slurries of Roberts et al. (1976). On investigation (Tompkin et al., 1978a), one reason was found to be the addition of sodium isoascorbate to the canned meat but not to the meat slurries. Isoascorbate had enhanced the antibotulinal efficacy of nitrite in the canned meat.

Christiansen et al. (1978) concluded that when inoculated, perishable canned cured meat is placed at 27°C, a race occurs between death of germinated botulinal cells and depletion of residual nitrite. Botulinal outgrowth was dependent on the relative levels of residual nitrite and surviving botulinal cells. This agreed with an earlier observation (Tompkin et al., 1978b) involving product held at 4.4°C or 10°C for up to half a year before placing at 27°C. The longer the product was held at refrigeration temperatures, the more the residual nitrite levels declined and the less inhibitory the product became when placed at 27°C.

In another study, thermal processing within the range of 63°C to 74°C (internal temperature) influenced neither residual nitrite levels nor botulinal inhibition in perishable canned cured meat

(Tompkin et al., 1978c). Adding hemoglobin, however, which resulted in a lower level of residual nitrite after processing, decreased botulinal inhibition. It was also shown that the type of meat could influence the degree of botulinal inhibition. Substituting turkey thigh meat, beef round, beef hearts, or pork hearts for fresh pork ham reduced the efficacy of nitrite.

The reason isoascorbate enhanced the antibotulinal effect of nitrite was pursued (Tompkin et al., 1978d). Isoascorbate, ascorbate, cysteine, and ethylenediaminetetraacetic acid (EDTA) were found to share a common function in meat, which later was demonstrated to be the sequestering of iron (Tompkin et al., 1978e). This made it possible to explain the differences in the relative efficacy of nitrite in various meats on the basis of their iron content, with one exception: beef liver. Despite its relatively high iron content (49 $\mu\text{g/g}$), beef liver did not cause a substantial loss in the antibotulinal efficacy of nitrite. In a later unpublished test with pork liver that has a much higher iron content than beef liver (225 to 243 versus 49 $\mu\text{g/g}$), no botulinal inhibition was observed. Subsequent research more clearly confirmed the relation of available iron to the antibotulinal efficacy of nitrite (Tompkin et al., 1979a).

Suggested mechanisms were proposed for the relation of iron to the antibotulinal efficacy of nitrite (Tompkin, 1978; Tompkin et al., 1978e). One possibility involved the reaction of nitrite with an essential iron-containing compound (e.g., ferredoxin) within the germinated cell. This was based on the rationale that in clostridia, nonheme iron-sulfur proteins (e.g., ferredoxin) are essential for electron transport, energy production, and enzyme activity. Clostridial ferredoxin is believed to contain two independent, single-electron transfer sites consisting of four iron atoms. Each active site is comprised of iron bonded to both cysteine and sulfur (Buchanan and Arnon, 1970). It was assumed that some form of nitrogen oxide-iron reaction could occur with the nonheme iron-sulfur proteins as occurs with nitric oxide and the iron in heme compounds (e.g., catalase, peroxidases, cytochromes, cytochrome oxidase, hemoglobin, and myoglobin). On the basis of the data existing in 1978, a simplistic explanation for the inhibition of clostridia consisted of the uptake of undissociated nitrous acid, a nitrogen oxide (e.g., nitric oxide)-iron reaction as in ferredoxin, and interference with energy metabolism in the vegetative cell to prevent outgrowth. Subsequent outgrowth could occur when (1) residual nitrite depletes to noninhibitory levels, allowing the dissociation of the nitrogen oxide from the iron, as can occur with nondenatured (i.e., nonheated) heme compounds, and/or (2) repair through the formation of new unreacted ferredoxin by the vegetative cell. A third possibility is the germination of dormant spores after residual nitrite has declined to noninhibitory levels.

Although isoascorbate enhances the antibotulinal effect of nitrite in freshly prepared perishable cured meat that is temperature abused, isoascorbate also reduces the efficacy of nitrite by causing more rapid depletion of residual nitrite (Tompkin et al., 1979b).

Wojton et al. (1978) studied the effect of nitrite on the stability of naturally contaminated perishable canned pork when temperature abused at 37°C. The product was formulated with 2.5% salt and sodium nitrite (0, 50, 100, or 200 $\mu\text{g/g}$) and then processed to an internal temperature of 69°C. As the level of nitrite decreased, the rate at which the product spoiled increased. Spoiled cans yielded *C. perfringens*, *C. sporogenes*, *Clostridium bifermentans*, *Bacillus* species, and enterococci.

The efficacy of nitrite in frankfurters was explored by Sofos et al. (1979b-e). Their test system mainly consisted of extruding a frankfurter emulsion into test tubes, heating to 68.5°C, overlaying with vaspar, cooling, and then storing the tubes at 27°C. This avoided the problem of recontamination inherent in some studies after heating, but as in other model systems receiving a mild heat treatment, a background flora of thermotolerant organisms existed from the ingredients. When testing a frankfurter emulsion prepared with mechanically deboned chicken meat, low levels of nitrite (20 and 40 $\mu\text{g/g}$) did not influence botulinal growth or toxin production (Sofos et al., 1979b). Adding 156 $\mu\text{g/g}$ of sodium nitrite delayed botulinal toxin production; however, the degree of inhibition was less than that reported by others.

Sofos et al. (1979c) evaluated various soy proteins in their system. The rate of botulinal toxin production in formulations with soy protein was equal to or slower than in an all-meat formulation, in both the presence and absence of nitrite. The type of soy protein influenced the results. Nitrite was ineffective in a formulation of soy isolate and pork fat. The depletion of residual nitrite was faster as the amount of beef was increased in a formulation containing soy. The rate of nitrite depletion was influenced by the type of soy.

Sofos et al. (1979d) compared the efficacy of nitrite (0 and 80 $\mu\text{g/g}$) in chicken, beef, and pork in their system. The addition of 80 $\mu\text{g/g}$ of sodium nitrite was relatively ineffective in chicken and beef. A significant delay in botulinal toxin production occurred in pork. The rate of botulinal toxin production was slower in all soy protein formulations than in all meat formulations. In a meat–soy mixture, the addition of 156 $\mu\text{g/g}$ of sodium nitrite was very effective in delaying botulinal toxin production.

Sofos et al. (1979e) found the rate of germination of *C. botulinum* spores in a chicken frank formulation to be unaffected by the addition of sodium nitrite (0, 20, 40, and 156 $\mu\text{g/g}$). They concluded that the effect of nitrite was on delaying the development of the cells after germination and before toxin was produced.

Vacuum-Packaged and Fermented Meats

The effect of nitrite in vacuum-packaged franks was studied on two occasions. In the first, Hustad et al. (1973) formulated franks with various levels of nitrite and nitrate, 400 $\mu\text{g/g}$ of sodium ascorbate, about 2.8% fermentable carbohydrate, and 4.52% brine. Adding 50 $\mu\text{g/g}$ of sodium nitrite delayed botulinal toxin formation for more than 4 weeks during abuse at 27°C, and then only 2 of the 55 franks became toxic. Although product made with 150 $\mu\text{g/g}$ of sodium nitrite did not become toxic, 450 $\mu\text{g/g}$ of nitrate gave a variable response. The strong inhibitory effect observed was likely the result of the combined effects of nitrite; the relatively high brine; and, more importantly, a fairly rapid decrease in product pH during abuse.

The second test, reported by Bowen and Deibel (1974) and Bowen et al. (1974), examined whether an increase in ascorbate over normal levels would alter the antibotulinal effect of nitrite. The franks were produced in the same facilities and formulated as before (Hustad et al., 1973), except for changes in the ratio of pork to beef, the levels of nitrite (0 to 150 $\mu\text{g/g}$) and ascorbate (9, 105, and 655 $\mu\text{g/g}$), and omission of nitrate. The finished franks had a brine of 4.83%. Toxic product occurred with 50 $\mu\text{g/g}$ of sodium nitrite at all levels of ascorbate. One toxic sample occurred at 100 $\mu\text{g/g}$ and none at 150 $\mu\text{g/g}$ of sodium nitrite. It was concluded that ascorbate did not affect the antibotulinal efficacy of nitrite. A later review of the pH data showed that all variables had a pH of 5.0 or less within 14 days, the exception being one variable with 150 $\mu\text{g/g}$ of nitrite. In that variable the pH was 5.0 or less within 21 days. Assuming a similar pH response in the first test (Hustad et al., 1973), the two studies indicate that the combination of 4.5% to 4.8% brine and nitrite levels of 50 to 100 $\mu\text{g/g}$ delayed botulinal outgrowth until the lactic flora decreased the pH to an inhibitory level. Under these circumstances, the conclusions in both studies concerning the inhibitory levels of nitrite, input versus residual nitrite, and the effect of ascorbate in franks should be restated or reexamined.

The decline in pH caused by a lactic fermentation was also the dominant factor preventing botulinal outgrowth in fermented sausage (Christiansen et al., 1975). In mildly heated (58.3°C) sausages containing dextrose, only those variables without nitrite became toxic at 27°C. The pH of product made with 50, 100, or 150 $\mu\text{g/g}$ of nitrite declined to 5.08 or lower within 1 week at 27°C, and the products remained nontoxic. Toxin was produced in all variables formulated without dextrose and more quickly in product having 0 or 50 $\mu\text{g/g}$ of nitrite. Toxin production was slower, and fewer toxic samples occurred in product with 100 or 150 $\mu\text{g/g}$ of nitrite. The pH of the dextrose-free product remained above 5.6.

A request was made by the poultry industry to the U.S. Food and Drug Administration (FDA) for approval of sodium nitrite as an additive in poultry products. The request included results by Christiansen et al. (1977) that demonstrated the efficacy of sodium nitrite in poultry meat. The data included botulinal challenge tests in frankfurters made with chicken meat and ham made with turkey meat. A summary of the data showing the antibotulinal effect of nitrite in the two poultry products appears in Hauschild (1982).

Raevuori (1975) studied the combined effect of sodium nitrite (0, 100, and 200 $\mu\text{g/g}$) and sodium erythorbate (0, 500, and 1000 $\mu\text{g/g}$) on the growth of *Bacillus cereus* in cooked sausage placed at 20°C for 48 hours. Erythorbate alone was not inhibitory. Erythorbate was found to enhance the inhibitory effect of nitrite. A combination of 200 $\mu\text{g/g}$ of sodium nitrite and 500 $\mu\text{g/g}$ of sodium erythorbate prevented the growth of the two strains of *B. cereus* tested. Erythorbate caused reductions in the residual nitrite and the redox potential of the product. Raevuori encouraged more widespread use of erythorbate for sausage production in Finland.

Omitting nitrite from commercially produced liver sausage in Finland led to rapid spoilage by gas-producing anaerobes and suggested a potential risk of botulism (Ala-Huikku et al., 1977). Inoculation studies examined the effect of nitrite (0 and 100 $\mu\text{g/g}$) and storage temperature (15°C, 20°C, and 25°C) on the rate of botulinal toxin formation. After cooking in Saran™ casings, the sausages had relatively high pH and water activity values of 6.65 to 6.80 and 0.98, respectively. Product without nitrite became toxic within 3 days at 25°C and within 6 days at 20°C and remained nontoxic through 14 days at 15°C. Product with 100 $\mu\text{g/g}$ of sodium nitrite remained nontoxic through 14 days at all three temperatures. Noninoculated product containing nitrite developed a slight unusual taste at 20°C owing to the growth of spore-forming spoilage organisms (e.g., *B. cereus*). The naturally occurring *B. cereus* was evidently more resistant to nitrite than *C. botulinum*.

Bacon

Crowther et al. (1976) in the United Kingdom studied the effects of a mixture of nitrite (100 or 200 $\mu\text{g/g}$) and nitrate (250 $\mu\text{g/g}$), ascorbate (0, 1000, and 2000 $\mu\text{g/g}$), and brine level (mild, 2.9% to 4.9%; medium 4.7% to 6.6%) on botulinal toxin production in vacuum-packaged back bacon. There was a general trend toward reduced residual nitrite levels at the time of inoculation when ascorbate was added. Because brine levels could not be held constant, the interaction of nitrite and ascorbate is difficult to interpret. When all samples having the mild and medium brine were compared, 42.7% and 18.9% became toxic, respectively. If all samples with no mixed cure, mixed cure with 100 $\mu\text{g/g}$ of nitrite, and mixed cure with 200 $\mu\text{g/g}$ of nitrite were compared, 67.4%, 19.3%, and 34.7% became toxic. If all samples containing mixed cure with and without ascorbate were compared, 35.8% and 21.9% became toxic, respectively. A higher percentage of samples were toxic with the addition of 200 $\mu\text{g/g}$ of nitrite than with 100 $\mu\text{g/g}$ of nitrite. The addition of ascorbate enhanced the antibotulinal effect of 100 $\mu\text{g/g}$ but not 200 $\mu\text{g/g}$ of nitrite. These values raise a question concerning the conclusions that (1) protection was greater if the level of nitrite was increased to 200 $\mu\text{g/g}$ and (2) sodium ascorbate at a level up to 2000 $\mu\text{g/g}$ did not reduce the protection afforded by nitrite against *C. botulinum*.

It also was reported that *S. aureus* grew well in the medium-salted bacon, regardless of the level of nitrite or ascorbate. As expected, growth was faster at 25°C than at 15°C. In opened packages, higher populations of *S. aureus* developed and enterotoxin A was produced. In vacuum packages, lower populations resulted and enterotoxin was not detected.

Shaw and Harding (1978) studied the effect of nitrate and nitrite on the microbial flora of Wiltshire bacon. The predominant flora of the bacon after curing consisted of micrococci, *Moraxella* species, and *Moraxella*-like bacteria. Omitting nitrate led to higher numbers of *Moraxella* species in the cured bacon. Bacon that was sliced and vacuum packaged developed a flora mainly of micrococci and lactics. Including nitrate in the bacon enhanced the growth of micrococci. The nitrate likely served as an electron acceptor for the growth of micrococci in the vacuum package.

Because higher numbers of lactics were present in bacon with the lowest initial nitrite concentration, it was suggested that nitrite could be important in delaying the sour spoilage caused by the growth of lactics.

The USDA conducted a test to verify the antibotulinal effectiveness of a combined nitrite–sorbate mixture in bacon (Johnston, 1979). Three types of bacon were produced in four commercial plants: bacon with no preservative, bacon with 120 µg/g of sodium nitrite, and bacon with 40 µg/g of sodium nitrite plus 0.26% potassium sorbate. All three bacons contained 550 µg/g of sodium ascorbate or sodium isoascorbate. The levels of sodium chloride, phosphate, and fermentable carbohydrate (e.g., sucrose) were left to the discretion of the producing plant. The bacon was shipped to a laboratory, inoculated with 1000 spores/g, and vacuum packaged (100 g per package). The bacon was then incubated at 27°C for up to 56 days. The results demonstrated that the combination of nitrite–sorbate was equal to or better than nitrite alone. There was considerable variation in toxin production in the bacon from the four plants. This was likely the result of the interaction of the other components of the product (brine level, fermentable carbohydrate, and growth of a spoilage flora). At least on the basis of the botulinal results, the data supported the potential approved use of a nitrite–sorbate blend to produce bacon.

A total of 30 botulinal studies were performed with vacuum-packaged bacon during the 1970s in the United States and Canada. They can be more readily compared because they were similar in processing and final product. Although the studies were performed for a variety of reasons, it is possible to glean the relative effect of nitrite by examining the nitrite-containing control variables from each test. Of the 30 tests, 10 appear in the technical literature (Christiansen et al., 1974; Bowen and Deibel, 1974; Collins-Thompson et al., 1974; Ivey et al., 1978; Sofos et al., 1980b; Tanaka et al., 1980).

The data indicate the following:

1. The pH of the bacon during abuse significantly influences the potential for botulinal outgrowth. Vacuum-packaged bacon prepared with 0.7% sugar (sucrose) or more provides sufficient fermentable carbohydrate that naturally occurring lactics cause a decline in pH to inhibitory levels.
2. Brine levels below 4.0% are not inhibitory to botulinal outgrowth. As the brine level exceeds 4.0%, outgrowth is increasingly delayed. If a lactic fermentation develops in the interim, the combination of relatively higher brine and decreasing pH can prevent botulinal outgrowth.
3. The level of residual nitrite at the time the bacon is abused influences the extent of the delay in botulinal outgrowth. The level of nitrite added to the product is not important, aside from the fact that the amount of added nitrite partially determines the level of residual nitrite.
4. The addition of ascorbate or isoascorbate can act in concert with residual nitrite to retard botulinal outgrowth in freshly produced bacon. However, ascorbate and isoascorbate can also have a negative effect by causing more rapid loss of residual nitrite during processing and storage.
5. Too high a level of botulinal cells when the bacon is abused can overwhelm the inhibitory system. The initial period of delay depends on the inoculum level.
6. Botulinal outgrowth is influenced by the temperature of abuse. Temperatures below 27°C or 30°C increase the delay in outgrowth and the inhibitory effects of the foregoing factors.
7. Because phosphates were present in almost all the studies, it is difficult to assess their effect. Within the levels and types used, phosphates do not appear to influence botulinal outgrowth in bacon.
8. These factors interact to influence botulinal inhibition.

Tests with *S. aureus* and Other Bacteria

A cluster of reports appeared during the early 1970s that dealt with the effect of nitrite on *S. aureus*. Collectively, the data along with the aforementioned results of Christiansen and Foster (1965) and Crowther et al. (1976) indicate that the growth of *S. aureus* is reduced and enterotoxin production can be prevented by the environmental conditions existing within vacuum-packaged cured meats. Reasons for the reduced growth and enterotoxin production are evident from the reports, but it remains debatable how much each of the possible factors (e.g., low oxygen level, nitrite, brine, growth of competitive flora, and product pH) contributes to this effect.

Nurmi and Turunen (1970) studied the effect of adding nitrite to a previously autoclaved broth medium (pH 6.0). Lactobacilli (78 strains), micrococci and staphylococci (24 strains), and *Pediococcus cerevisiae* (1 strain) were examined for their tolerance to nitrite in the presence and absence of 4.01% salt. Very high levels of nitrite (1000 and 5000 µg/g) either prevented or severely retarded the growth of all strains. At 200 µg/g growth was delayed or slower. At 40 µg/g growth was comparable to that in the control without nitrite, 8 µg/g enhanced the growth of the lactobacilli and, to a slight extent, the micrococci. Heterofermentative lactobacilli were more tolerant of salt than the homofermentative strains.

Genigeorgis et al. (1969) found the production of type B staphylococcal enterotoxin to occur earlier on ham at 30°C than at 22°C to 24°C and earlier under aerobic than anaerobic conditions. The commercially canned ham used for the test had a brine level of 3.6%, no residual nitrite, and an abnormally low pH of 5.3. Extensive tests were also reported for laboratory-cured ham, but it is unclear whether nitrite level influenced anaerobic enterotoxin production. Toxin was produced in certain variables having residual nitrite levels as high as 145 µg/g. The probability of toxin production at 10°C reportedly decreased as the level of nitrous acid increased. Nitrous acid levels were calculated from the residual nitrite and pH values as described by Castellani and Niven (1955). Anaerobic toxin production was prevented with 0.6 µg/g or more nitrous acid and pH values below 5.58. However, because high nitrous acid values (>0.6 µg/g) were possible only at reduced pH levels (pH 5.6 or less), it is uncertain whether high nitrous acid content, reduced pH, or both prevented anaerobic toxin production. At higher temperatures (22°C and 30°C), anaerobic toxin production occurred at pH 5.3 in two samples with negligible nitrite levels. A third sample with a pH of 5.34, 22 µg/g of nitrite, and 0.159 µg/g of nitrous acid also became toxic.

Buchanan and Solberg (1972) studied the effect of nitrite (0, 200, 500, 1000, and 2000 µg/g), oxygen pressure, and initial pH (6.3, 7.3) on the growth of *S. aureus* in brain heart infusion broth (BHI). At pH 7.3, nitrite was without effect in an aerobic environment and 500 µg/g or less was without effect anaerobically. At pH 6.3 and 200 µg/g, the increase in the adjustment phase was 10 hours or less aerobically and about 30 hours anaerobically. The authors questioned the hypothesis that nitrite inhibition is dependent on the conversion of nitrite to nitrous acid. Nitrite was metabolized by *S. aureus* under aerobic but not anaerobic conditions. This suggested either that nitrite was not being used as an electron acceptor or the nitrite reductase system was blocked anaerobically. After considering the effects of changing pH during the growth of *S. aureus* and a review of the literature, Buchanan and Solberg suggested that nitrite inhibits the growth of *S. aureus* by blocking the sulfhydryl sites of either coenzyme A or α -lipoic acid, thus preventing the normal metabolism of pyruvate. They also concluded that vacuum packaging should improve the safety of cured meats relative to *S. aureus*.

Of significant importance was a report that the growth of *S. aureus* was found to be restricted to the periphery of commercially fermented sausage (Barber and Deibel, 1972). Understanding why this occurs would be very instructive. One reason given was that *S. aureus* is less acid tolerant under anaerobic conditions; however, future research might demonstrate the involvement of additional factors. Inoculated raw sausage (2.9% salt and 100 µg/g of sodium nitrite) incubated at 37°C in environments of 20%, 15%, 10%, 5%, and 0% oxygen yielded lower *S. aureus* populations as

the oxygen level decreased. Although adequate growth occurred within 48 hours, enterotoxin was not detected through 5 days of storage in the absence of oxygen.

Markus and Silverman (1970) reported enterotoxin A production in broth media (pH 6.6 to 6.8) to be unaffected by sodium nitrite (200 µg/ml) or sodium nitrate (1000 µg/ml). Perhaps because of the atypically high pH, no synergistic effect occurred between salt, nitrite, and nitrate on either growth or enterotoxin production. Contrasting results were subsequently reported that showed the production of enterotoxin A to decrease as pH decreased, salt increased, and nitrite increased (Tompkin et al., 1973).

Morse and Mah (1973) studied the effect of glucose on enterotoxin B synthesis in a broth medium buffered to an alkaline pH (7.7). Adding glucose caused decreased toxin production as a result of the rapid oxidative decarboxylation of pyruvate. Subjecting the cells to anaerobic shock (sparging with 95% N₂ and 5% CO₂) to inhibit pyruvate decarboxylation caused an increase in the rate and quantity of toxin produced; the percentage of lactic acid as an end product; and, presumably, more available energy. Adding nitrite as an electron acceptor at the time of anaerobic shock prevented increased toxin production, and nitrate was converted to nitrite. Glucose repression of enterotoxin B production was also reported to occur at pH 6.0 but to a lesser degree than at pH 7.7 (Morse and Baldwin, 1973).

Bean and Roberts (1974, 1975) demonstrated the combined effect of pH, salt level, and nitrite on the heat resistance and recovery of *S. aureus*. There was a general pattern of increased heat resistance as salt level (0, 4, and 8%) increased at pH 6, 6.5, and 7.0 but not at pH 5.5 or 5.0. The effect of nitrite on heat resistance was inconsistent. However, when added to the recovery medium, increasing the level of nitrite had the general effect of reducing the recovery of both heat-damaged and undamaged cells. The inhibitory effect of nitrite in the recovery medium increased with increasing salt content, decreasing incubation temperature, and decreasing pH.

In another report on non-spore-forming bacteria, Rowe et al. (1979) reported that nitrite inhibits active transport, oxygen uptake, and oxidative phosphorylation in *Pseudomonas aeruginosa*. The evidence strongly suggested that nitrite exerted its inhibitory effect by oxidizing ferrous ion of an electron carrier(s) (e.g., cytochrome oxidase) to the ferric state. Nitrite did not affect glucose transport by *S. faecalis* or *Streptococcus lactis*, both of which lack cytochromes. They instead rely on glycolysis for generating adenosine triphosphate (ATP) when grown on glucose and transport glucose via phosphoenolpyruvate-phosphotransferase rather than by active transport.

SUMMARY FOR 1970–1980

The Perigo Factor in Cured Meats

1. The formation of the Perigo factor in commercially cured meat is highly debatable. Formation of some similar inhibitor(s) (PTF) during the thermal processing of shelf-stable canned cured meat appears possible. The data of Pivnick and Chang (1974) are convincing evidence that a small percentage of the total inhibition can occur that is not the result of residual nitrite *per se*.
2. It has been difficult to design tests that prove the presence or absence of an unknown inhibitor that is likely unstable and is apparently formed in low quantity and functions within the midst of a complex system (e.g., salt, residual nitrite, and pH), the sum of which influences stability. If manipulations of thermally processed meat were attempted to extract such an inhibitor, the question could be raised whether the inhibitor would be altered or destroyed.

The Perigo Factor in Media

The data appear conclusive that nitrite, iron, and sulfhydryl groups are involved in the formation of the inhibitor.

Perishable Cured Meats

Tests on a variety of cured meats showed the efficacy of nitrite to be highly variable among the laboratories and the products tested.

The list of recognized factors influencing the antibotulinal efficacy of nitrite continued to grow and included the following:

1. pH of the product during abuse
2. Brine level
3. Residual nitrite upon abuse and the rate of depletion during abuse
4. Level of viable botulinal spores and vegetative cells at the time of abuse
5. Temperature of abuse
6. Level of ascorbate or isoascorbate
7. Level of "available" iron in the product
8. Type of meat and other formulation ingredients
9. The thermal process applied to the product
10. The growth of competitive flora
11. The level and type of phosphate might play a role through their influence on product pH, but this effect was not clearly defined in meats. Whether phosphates can contribute to inhibition by some other mechanism (e.g., sequestering iron) remained unclear.

All the factors are interrelated. Although the thermal process given to perishable cured meats does not appear to cause thermal injury to the spores, long, slow, heating times, as used in certain experiments with bacon and large-diameter sausages, can provide the opportunity for germination followed by destruction, and perhaps injury, of the germinated cells as the temperature is increased.

Inhibition seems to be influenced only by the amount of residual nitrite in excess of the asymptotic level on the depletion curve. The low level of residual nitrite that continues to be detected after prolonged storage does not appear to be inhibitory. The disappearance of residual nitrite in cured meat is faster as product pH is decreased and isoascorbate level and storage temperature are increased. Destruction of residual nitrite could occur directly by utilization of nitrite by certain microbes and perhaps indirectly by reaction with degradation products produced during the growth of the flora. In addition, certain competitive flora are inhibitory to botulinal outgrowth (e.g., enterococci). PTFs do not appear to play any role in perishable cured meats.

Mechanism of Nitrite Inhibition

1. Inhibition of *C. perfringens* and *S. aureus* was suggested to involve the blocking of sulfhydryl sites within the bacterial cells.
2. Nitrite was reported to inhibit active transport, oxygen uptake, and oxidative phosphorylation in *P. aeruginosa*. The effect appeared to be at the electron carrier level. Glucose transport was not affected in two bacterial species that lack cytochromes.
3. It was suggested that nitrite inhibition of *C. botulinum* may be the result of a reaction of nitric oxide with an essential iron-containing compound (e.g., ferredoxin) within the germinated cells.

1980–1990

The National Academy of Sciences (NAS) examined the health effects of dietary nitrate and nitrite and evaluated possible alternatives to the use of nitrite as a preservative in food (Assembly of Life Sciences, 1981, 1982). The investigation was conducted at the request of the FDA and the USDA. The NAS conclusions were then used to determine regulatory policy for the use of nitrite and nitrate in foods. The recommendations are also important because they influenced the intensity and direction of research. Following is a summary of the 11 points in the recommendations:

1. Results of limited experiments suggest that nitrate is neither carcinogenic nor mutagenic.
2. Evidence does not indicate that nitrite acts directly as a carcinogen in animals.
3. Most N-nitroso compounds (NOCs) are carcinogenic in laboratory animals and mutagenic in microbial and mammalian test systems, and some are teratogenic in laboratory animals.
4. The exposure of humans to nitrite and nitrate should be reduced. Exposure to nitrite should be reduced to the extent that protection against botulism is not compromised. Except for its use in dry-cured and fermented products, the use of nitrate should be discontinued in meat and poultry products. Attention should be given to reducing the nitrate content of vegetables and drinking water.
5. Environmental exposure to NOCs should be determined, and methods to reduce exposure should be developed. Improved methods are needed to distinguish between free and bound nitrite and whether residual nitrite is a true measure of nitrosating capacity.
6. Sources of human exposure to amines and nitrosamines (e.g., pesticides and drugs) should be modified to reduce exposure. Amino compounds that can be nitrosated *in vivo* should be identified and investigated.
7. Additional research is needed on the metabolism and pharmacokinetics of nitrate in humans. This includes the role of bacteria in the reduction of nitrate to nitrite and NOC formation, especially in certain clinical conditions.
8. Ascorbate is now added to bacon to inhibit the formation of nitrosamines. Further research is needed to determine the amount of nitrite that is destroyed in the human stomach and the extent to which nitrosation reactions are modified by various inhibitors. Interactions among inhibitors, catalysts, and other dietary components deserve attention.
9. Further studies are required to determine the mechanisms whereby nitrite controls the outgrowth of *C. botulinum* spores, its action in cured meats, its antioxidant activity, and its effect against spoilage microorganisms and pathogens other than *C. botulinum*.
10. Although it is not possible to estimate the potential morbidity or mortality from *C. botulinum* if nitrite were removed as a curing agent from certain products, for public health safety it is prudent to consider that certain preserved foods may contain *C. botulinum* and may be abused.
11. In view of the possible but unquantified risk resulting from the use of nitrite as a curing agent, the search for alternatives to the use of nitrite should be continued. No new agent or combination of agents should be used to replace nitrite until the safety of the new agent(s) has been ensured.

If the NAS committee had concluded that nitrite is carcinogenic, the regulatory agencies would have had to disapprove the use of nitrite as a food additive. Such action would have eliminated all cured meat and poultry products from the market.

The momentum established in the previous decade led to new information on the possible mechanisms involved in nitrite inhibition. Although there was continued emphasis on the anti-clostridial effect of nitrite, there was also interest in other microorganisms. Research on alternative agents and/or processing methods to replace nitrite as a preservative was actively pursued. Concern

for sodium intake in the diet brought new pressure to reduce the salt content of cured meats, further accelerating the trend of the past 40 years. Considering the recent reductions in the amount of nitrite used for curing meats and the voluntary elimination of nitrate from most cured meats in the United States, the impact of further reductions in salt deserved close examination. Another major trend developed during this time. Throughout the decade, the per capita consumption of red meats decreased while the consumption of poultry products increased. In 1983, the FDA acknowledged that prior sanction existed for the use of nitrite as an additive to poultry meat (Cassens, 1990). Since that time, there has been a very rapid growth in the volume and variety of cured poultry products in the United States.

Assessment of the Botulinal Risk in Cured Meats

Hauschild (1982) summarized the existing data from botulinal challenge tests in a wide variety of meat products. He proposed expressing botulinal inhibition as $\log I/P$, where P is the probability of outgrowth and toxigenesis from one spore. This was used to quantitatively compare the data from the various challenge tests. The data provided estimates of the relative degree of botulinal risk for each product. The level of risk differed among the various products. Bacon was considered a low-risk product, whereas turkey ham was a high-risk product. The level of risk could also differ among products of the same type as a result of differences in their brine concentrations. He concluded that significant reductions in nitrite should not be made for all cured meats. However, selective reductions in nitrite could safely be made provided the levels of brine, carbohydrate, and erythorbate are controlled.

Hauschild and Simonsen (1985) conducted an extensive review of the technology for producing shelf-stable canned cured meats (Table 6.1). Their review was to assist in the development of a document for these products. They summarized research, existing government regulations, and commercial practices to arrive at guidelines to assure the microbiological safety of these products. They recommended that the bacterial spore levels in the raw product be rigidly controlled and that 150 $\mu\text{g/g}$ of sodium nitrite be used to produce products with the following brine and thermoprocess values.

Reductions in the recommended levels should be based on extensive plant experience and/or experimental work and controls to assure minimal spore levels. Reductions in the level of nitrite can be compensated for by moderate increases in the brine level and/or thermoprocess. This information was reiterated by Hauschild and Simonsen (1986).

TABLE 6.1
Shelf Stable Canned Cured Meats: Minimal Brine and Thermoprocess with 150 $\mu\text{g/g}$ Sodium Nitrite

Product	% Brine	Thermoprocess (F_0)
Luncheon meat	3–4	1.0–1.5
	4.0–4.5	1.0
	4.5–5.0	0.5–1.0
	5.0–5.5	0.5
Ham and shoulder	3.3	0.3–0.5
	3.7	0.2–0.3
	4	0.1–0.2
Sausages	2.5	1.5

Source: Hauschild and Simonsen (1985).

Botulinal Challenge Tests

Tests in meat and fish products. Toxin production by a mixture of type E and nonproteolytic type B botulinal strains in liver sausage, fermented sausage, and hams was investigated by Lücke et al. (1980). Liver sausage was prepared with 83 µg/g of added sodium nitrite, pH 6.2, and a_w 0.98. Toxin was not produced at 5°C. As temperature was increased to 8°C, 10°C, and 15°C, toxin production was increasingly more rapid. In fermented sausage, the inhibitory effect of the lactic fermentation and decreasing water activity values during fermentation and subsequent drying prevented botulinal toxicity. Large bone-in hams were inoculated with nonproteolytic botulinal spores before curing in a solution of salt, nitrite, and nitrate at 8°C. Toxin was detected after 9 to 11 days. As the hams continued through the completion of curing, toxin levels remained high, but the recovery of viable botulinal cells became increasingly difficult. After curing, toxin was detectable 10 cm from the injection site. The hams had a slight off-odor but no apparent putrefaction. Similar results were obtained during the dry curing of hams at 8°C or 10°C. They concluded that nonproteolytic, psychrotrophic strains can grow and produce toxin during the early stages of curing before the salt and nitrite levels penetrate and reach inhibitory levels. Additional data on the ham experiments were presented by Lücke et al. (1982). Methods and media were recommended to facilitate the recovery of both the proteolytic and the psychrotrophic nonproteolytic strains of *C. botulinum*. The latter were suspected to be of particular importance as a cause of botulism from raw cured meat products in Europe (Lücke, 1982).

Hauschild et al. (1982) assessed the relative risk of botulism from temperature-abused liver sausage. At brine levels of 3.8% to 4.2%, nitrite in the range of 0 to 100 µg/g had little or no effect on toxigenesis. Greater protection was obtained with 150 µg/g. At a higher brine level of 5.44%, nitrite was more effective and an appreciable delay in toxicity was obtained with 50 and 100 µg/g of nitrite. Product with low levels of toxin often appeared acceptable. Storage at 8°C until a nearly complete loss of nitrite occurred did not diminish the effect of the nitrite. By increasing the processing temperature and/or prolonging the time of processing, the inhibitory effect of the nitrite was reduced. Another possibility could be offered for this observation. Perhaps the milder thermal process permitted the survival of a competitive flora that prevented toxigenesis. By increasing the thermal process, competitive flora could have been destroyed, thereby permitting the more heat resistant *C. botulinum* to dominate and develop toxin. Hauschild and Hilsheimer (1983) examined 138 samples of liver sausage from retail outlets in Canada. The number of botulinal spores was estimated to be about 0.15 spores/kg in the product. The total *C. botulinum* was estimated to be about 0.24/kg.

The use of nitrite in smoked fish in the United States has been restricted. To prevent the growth of *C. botulinum*, a high level of salt (5% brine) has been required. Some consumers find this amount of salt unacceptable. Cuppett et al. (1987) demonstrated that adding sodium nitrite to obtain an initial level of 156 µg/g could reduce the amount of salt needed for botulinal protection. The tests involved vacuum-packaged smoked whitefish fillets prepared with about 2.3%, 4.4%, and 6.2% brine. The pH of the fillets decreased during 27°C incubation from about pH 6.4 to as low as 5.2. The rate of pH decrease was slower as the brine level increased.

Tests in model systems. Sofos et al. (1980a) studied the significance of pH on the efficacy of nitrite and sorbic acid mixtures in a chicken frank emulsion. The test system consisted of a frank emulsion prepared from mechanically deboned chicken meat that was extruded into test tubes, heated, cooled, overlaid with vaspar, and incubated at 27°C. The antibotulinal efficacy of sorbic acid was pH dependent, with levels of at least below pH 6.0 being required. If a low level of nitrite (40 µg/g) was added, the pH of the formulation could be increased to pH 6.2 without loss of the benefit of sorbic acid. The nitrite (40 µg/g) also significantly increased the inhibitory effect of the sorbic acid. The presence of sorbic acid slowed the rate of nitrite depletion.

Wagner and Busta (1983) compared the antibotulinal effect of combinations of sodium nitrite (40 and 120 µg/g), sodium acid pyrophosphate (0% and 0.4%), and potassium sorbate (0% and

0.26%) in frankfurters. They used the same test system as in Sofos et al. (1979b–e, 1980a). Tubes showing gas or other spoilage were preferentially selected for toxin assay. The pH of the four product variables was between 5.7 and 6.0. The test system avoided the problem of recontamination that occurs when peeling and packaging franks. The combination of 40 µg/g of sodium nitrite, 0.26% potassium sorbate, and 0.4% sodium acid pyrophosphate caused the greatest delay in toxin production. There appeared to be more botulinal inhibition with the addition of sodium acid pyrophosphate.

Nelson et al. (1983) assessed the antibotulinal effect of three phosphates in the test system just described (Wagner and Busta, 1983). The frankfurters were made from chicken instead of beef and pork. The initial pH for the various products was 5.78 to 6.19. Polyphosphate increased botulinal inhibition when added to combinations of sorbic acid or potassium sorbate and a low level of nitrite (40 µg/g). Of the polyphosphates tested, sodium acid pyrophosphate was more effective than sodium hexametaphosphate or sodium tripolyphosphate. The polyphosphates did not influence botulinal inhibition in the absence of nitrite. The combination of nitrite (40 µg/g), potassium sorbate (0.26%), sodium acid pyrophosphate (0.4%), and pH control of the emulsion was the most effective to achieve reduced nitrite levels and provide enhanced botulinal inhibition. The pH was important in all treatments tested.

Jarvis et al. (1979) modified their pork slurry to a thicker consistency, more like that of a reformed ham after heating. They found that increasing the heating from $P_{80^{\circ}\text{C}} = 0.65$ to $P_{80^{\circ}\text{C}} = 6.65$, nitrite in the range of 175 to 300 µg/g was even more inhibitory to botulinal outgrowth as measured by the percentage of slurries that became toxic. This increased inhibition was not observed with nitrite levels of 75 to 125 µg/g. They also found that the antibotulinal efficacy of nitrite could be increased by the addition of phosphate, with certain phosphates being more effective than others.

Roberts et al. (1981a,b) evaluated the interactions of salt, nitrite, nitrate, isoascorbate, polyphosphate, and thermal process on botulinal outgrowth in pork slurries of two different pH ranges (pH 5.4 to 6.36 and 6.27 to 6.72). Statistically significant reductions in toxin production occurred with increasing salt level, nitrite level, or thermal process; adding isoascorbate or nitrate; or decreasing the abuse temperature. The pH of the pork used for the lower pH slurries ranged from 5.6 to 6.03, except for two outliers of 5.4 and 5.54. The pH of the pork used for the higher pH slurries ranged from 6.27 to 6.56. The addition of polyphosphate caused an increase of about 0.4 units in the lower pH slurries and about 0.1 to 0.2 units in the higher pH slurries. The increase in pH could explain the decreased inhibition in the lower pH slurries, but the reason for the increased inhibition when adding polyphosphate to the higher pH meat slurries is less evident. The levels of salt found in the slurries were often as much as 0.5% (wt/vol water) below the targeted values. It is unclear whether the target or analytical values for salt were used for statistical analysis, although the conclusions were stated on the basis of the target values of 2.5%, 3.5%, and 4.5% salt (wt/vol water). This may alter the quantitative assessment of the individual and combined effects of the additives, although the trends in the data may not change.

Roberts et al. (1981c) analyzed results from large multifactorial pork slurry tests to arrive at formulas for estimating the probability of toxin formation in their pork slurry system. A separate formula was developed for slurries prepared with low-pH pork (pH 5.5 to 6.3) and high-pH pork (pH 6.3 to 6.7).

Gibson et al. (1982) examined the effect of source of the meat on the antibotulinal effect of 100 µg/g of sodium nitrite in a pork slurry system. They found the nitrite to be less effective in slurries prepared with meat from the shoulder than meat from the leg. No systematic difference in inhibition was observed with the meat from three breeds of pig. Considerable variation occurred between the meat from different animals within each breed.

Roberts et al. (1982) examined the interaction of potassium sorbate in their cured pork slurry system. One nitrite level (40 µg/g) was used throughout. At a given spore inoculum, potassium sorbate significantly decreased toxin production. The effect of sorbate was greater as salt levels

increased (2.5% to 3.5%), as pH levels decreased to below 6.0, and as the storage temperature decreased.

U.K. legislation was proposed to control the use of nitrite by monitoring the residual nitrite in cured meat products. This led Gibson et al. (1984) to examine the effect of various factors on the rate of nitrite depletion and botulinal outgrowth. They found that the rate of depletion in their pork slurry system was influenced by many intrinsic and extrinsic factors. They concluded that measuring the residual nitrite of a product cannot be used to assess compliance with the nitrite regulations unless the analysis is performed soon after manufacturing the product. As expected, the rate of nitrite depletion was influenced by storage temperature, the addition of nitrate, and the addition of isoascorbate. The significance of microbial growth on nitrite depletion in the slurries during storage was not addressed. A rather high level of isoascorbate (1000 µg/g) caused nitrite to disappear rapidly and delayed the outgrowth of *C. botulinum*. They concluded that the level of residual nitrite did not influence botulinal outgrowth in their experiments. No suggestion was offered for the increased botulinal inhibition when nitrate was added. The addition of nitrate led to higher residual nitrite levels for a longer period.

The primary objective of the Bristol research group was to arrive at a model system to quantitate the effect of the various factors influencing botulinal inhibition in cured meats. Their approach was to conduct large multifactorial experiments using pork slurries and then to analyze the data statistically and develop equations that could be used for predictive modeling. The results are intriguing and leave relatively unexplored the underlying mechanisms influencing the results. For example, why was a high heat treatment (80°C for 7 minutes followed by additional heating at 70°C for 1 hour) more effective for increasing botulinal inhibition? Why should the initial pH of the meat influence the degree of botulinal inhibition?

Szczawinski et al. (1989) prepared ground pork slurries with 0, 50, 100, 156, and 200 µg/g of sodium nitrite. The slurries were irradiated, then inoculated with botulinal spores, sealed with vaspar, and stored at 30°C for 28 days. Residual nitrite levels after irradiation were inversely related to the irradiation dose. At lower dose levels of 0 to 9 kGy, there was a slight reduction of residual nitrite and the probability of botulinal outgrowth increased in only one variable (50 µg/g of sodium nitrite and the lower spore inoculum level). The probability of botulinal outgrowth decreased as the level of added nitrite increased above 50 µg/g. After higher doses of irradiation (10, 20, 30, 40, and 50 kGy), there was a marked decrease in the residual nitrite, which resulted in rapid botulinal outgrowth.

Perigo Factor

Jonsson and Raa (1980) heated cysteine at pH 6 to produce bis-(2-amino-2-carboxyethyl) trisulfide (Bactin) and found it to be bacteriostatic to *Lactobacillus plantarum*. Bactin was not produced when heating cysteine, unless ferric chloride was present and the pH was 6. The bacteriostatic action was reversed by the addition of cysteine. The authors suggested that Bactin formation may be responsible for the inhibition observed in Perigo-type media. They suggested that the bacteriostatic effect is the result of facilitated conversion of cysteine to Bactin and a concomitant removal of thio compounds, which otherwise prevent bacteriostatic activity.

In another study of Perigo factor formation, Custer and Hansen (1980) reported that lactoferrin, an iron-binding protein in casein, forms an inhibitor when autoclaved in the presence of nitrite, thioglycollate, and iron in culture media. Transferrin, a similar protein in blood serum, responded similarly. Pretreatment of the lactoferrin and transferrin with a protease was necessary for inhibitor formation. The amount of iron required for inhibitor formation was reportedly similar to the level found in muscle tissue. Higher or lower levels prevented the inhibitory effect. They concluded that lactoferrin and transferrin are precursors of the Perigo factor and that it is likely that nitrite-modified transferrin accounts for some of the antimicrobial effects of nitrite in cured meats. However, the

requirements of pretreatment with protease and autoclaving reduce the likelihood that this would occur under natural conditions in meats.

A PTF was produced when tryptone was heated with nitrite at 121°C for 20 minutes (Miwa and Matsuura, 1983). Inhibition was not observed when casein or an acid hydrolysate of tryptone was used. The maximum inhibitory activity was achieved by heating the medium containing 4% tryptone and 0.2% thioglycollate with more than 50 µg/g of sodium nitrite. There was evidence that the factor may be unstable toward oxygen. The factor was inhibitory against *S. aureus*, *B. subtilis*, and *C. botulinum* but not against *E. coli* or *S. Typhimurium*.

Hansen and coworkers published a series of papers devoted to the Perigo factor with the intent of learning the mechanism of nitrite inhibition. *B. cereus* was used throughout the research. They concluded that nitrite inhibits the germination of spores of *B. cereus* by inactivating membrane sulfhydryl groups (Buchman and Hansen, 1987). The relevance of this information to the effect of nitrite on *C. botulinum* and the stability of cured meats that are mildly heated remains to be established.

Alternatives to Nitrite for Botulinal Protection

A considerable amount of research has been devoted to finding an acceptable replacement for nitrite. The NAS report (Assembly of Life Sciences, 1982) is the most complete assessment of the possible replacements and the issues involved in finding a replacement. The committee considered the different functions of nitrite as a food additive (e.g., antioxidant, antimicrobial agent, or cured color and flavor development). In addition, a listing of some of the proposed chemical alternatives along with references was later provided by Roberts and Gibson (1986). None of the chemicals investigated have shown promise to date.

Bacon was identified as the one cured meat in which unacceptable levels of nitrosamine could be produced. Thus, special requirements were established by the USDA for the production of bacon and verifying compliance with a nitrosamine limit. When bacon is fried, the high temperature can result in the formation of NOCs. It was learned that the addition of ascorbate or isoascorbate (erythorbate) can block nitrosamine formation during the frying process. This led the USDA to reduce the input level of sodium nitrite to a maximum of 120 µg/g and to require the addition of 550 µg/g of either ascorbate or isoascorbate (Code of Federal Regulations, 2002a). Subsequently, two additional options were added to the USDA regulations (Code of Federal Regulations, 2002a). One option is to use a combination of 100 µg/g of sodium nitrite, 550 µg/g of sodium ascorbate or isoascorbate, and a USDA-approved partial quality-control program to assure compliance. The second option is to manufacture the bacon using a combination of 40 to 80 µg/g of sodium nitrite, a readily fermentable carbohydrate (i.e., a minimum of 0.7% sucrose), and a lactic acid-producing bacterial culture (i.e., *Pediococcus acidilactici*). The rationale for this approach is apparent from the summary outlined earlier in this chapter, in the bacon section for 1970–1980. The second option in the USDA regulation is based on a three-plant study that verified the efficacy of this concept (Tanaka et al., 1985). The research and subsequent regulation ignore the significance of brine level as an important factor that should also be controlled. The process also does not acknowledge the potential value of the natural contaminating flora associated with most bacon processing environments. If the brine level is controlled, an adequate quantity of fermentable carbohydrate is present, and sufficient sodium nitrite is added to produce an acceptable cured product, then the naturally occurring lactic flora would reduce the pH of the product to inhibitory levels in a high percentage of the packages if the bacon were temperature abused. A key to this approach is to not eliminate the lactics from the environment through an overly aggressive sanitation program.

A new approach to replace nitrite was developed in Canada. Nitrite is unique in being able to produce cured meats with the proper attributes of color, oxidative stability, flavor, and microbial stability. The system was developed to avoid the addition of nitrite. The system consists of using nitric oxide to cure the heme portion of beef blood. This cured pigment is then further processed,

dried, and then microencapsulated to provide stability against degradation by oxygen and light. The cured meat pigment would then be added, along with other ingredients (e.g., ascorbate, phosphate, and tertiary butylhydroquinone), to provide oxidative stability. The proposed system also requires the addition of an antimicrobial agent (e.g., sodium hypophosphite). O'Boyle et al. (1990) and Shahidi and Pegg (1992) have summarized the system and provided the status on the various aspects of the research.

Ismaiel and Pierson (1990) demonstrated that origanum oil, alone or in combination with sodium nitrite, could prevent botulinal outgrowth in a broth medium. The two additives acted synergistically. This effect was explainable by their modes of action. The origanum oil affects germination and vegetative growth; sodium nitrite affects outgrowth and vegetative growth. Unfortunately, origanum oil was not as effective when tested in pork. This was thought to be the result of the highly soluble oil components being absorbed into the lipid fraction of the meat. At the lowest spore levels tested, there was a consistent trend, although statistically insignificant, toward increasing inhibition with an increasing level of origanum oil in three of the four sets of nitrite levels. Perhaps the results would be more favorable if challenged with a lower botulinal spore level.

Mechanism of Nitrite Inhibition

Yarbrough et al. (1980) reported nitrite inhibition of oxygen uptake and proton-dependent proline transport in *E. coli*. Because glucose transport was not affected, this suggested that nitrite was inhibiting glucose catabolism. Nitrite was found to inhibit aldolase, but hexokinase was not affected. The authors concluded that nitrite inhibits bacteria by several mechanisms. First, nitrite interferes with energy conservation by inhibiting oxygen uptake, oxidative phosphorylation, and proton-dependent active transport. Second, nitrite acts as an uncoupler, causing a collapse of the proton gradient. Third, certain metabolic enzymes (e.g., aldolase) are inhibited. Because streptococcal aldolase was sensitive to nitrite but glucose accumulation was unaffected, the authors suggested the high resistance of *S. lactis* and *S. faecalis* is the result of the impermeability to nitrite in these bacteria.

Glucose and 2-deoxyglucose transport and accumulation by *S. aureus* have been reported to be inhibited by nitrite under anaerobic conditions (Simone and Solberg, 1981). The accumulation of glucose decreased in relation to increasing the nitrite level and the time of exposure to nitrite before adding sugar.

Palumbo and Smith (1982) found heat-injured *S. aureus* to undergo repair and grow in a nitrite-containing agar medium at pH 5.6. Recovery of the heat-injured cells was comparable in media with no nitrite and media containing 100 µg/g of sodium nitrite. At levels of 200, 400, and 800 µg/g, the recovery was progressively lower.

Fang et al. (1985) investigated the effect of nitrite on *S. aureus*. The inhibitory effect of nitrite was enhanced by applying stricter anaerobic conditions. Under less rigid conditions of anaerobiosis, *S. aureus* was more resistant to the nitrite and also caused a more rapid depletion of nitrite in the medium. The disappearance of nitrite was faster with *S. aureus*. The mechanism by which *S. aureus* hastens the loss of nitrite was not attributable to nitrite reductase activity, interactions with metabolic products, dismutation resulting from the reduced pH during growth, or absorption to the cells. Nitrite, nitric oxide, nitrous oxide, and ammonia were not end products of the sodium nitrite disappearance. The increased efficacy of nitrite under strict anaerobic conditions could be a factor affecting the growth of other microbes in cured meat and poultry products.

Kanner and Juven (1980) compared the anticlostridial effect of nitrite and S-nitrosocysteine (RSNO), which is formed during the curing of meats. They found that RSNO was less effective than nitrite for preventing the recovery of heat-shocked *C. sporogenes* spores. When salt was also present in the recovery medium, very little synergistic effect was observed with RSNO and salt, but a high level of synergism was observed with nitrite and salt. This suggested that RSNO is not the major anticlostridial factor in cured meat. The anticlostridial effect of RSNO increased as the

pH of the culture medium was lowered from 6.4 to 5.6. On an equimolar basis, RSNO (540 µg/g) was considerably less inhibitory than sodium nitrite (200 µg/g) when tested in inoculated perishable canned turkey at 27°C.

Chumney and Adams (1980) found *C. perfringens* spores to be sensitive to inhibition by salt, nitrite, and a mixture of polymyxin and neomycin after heat injury at 70°C to 100°C. They suggested the increased sensitivity to each agent was caused by the same cellular damage. Recovery of heat-injured spores in a basal medium containing 3% salt or 1% salt with 200 µg/g of nitrite yielded comparable recovery rates. Compared with earlier research, the nitrite was relatively ineffective as an inhibitor. This may have been the result of sulfite in the basal medium (tryptone sulfite neomycin agar) because sulfite destroys nitrite (Tompkin et al., 1980).

Collinge et al. (1981) reported that ferric chloride or hemoglobin stimulates the growth of *C. botulinum* (as opposed to reducing the antibotulinal efficacy of nitrite) in canned cured meat. They considered that nitrite inhibits *C. botulinum* by making the iron of the heme groups in meat unavailable for growth. Adding nitrosylated myoglobin to the meat system resulted in a rather inhibitory system compared with adding untreated myoglobin. Also, there was no noticeable relation between botulinal growth rate and residual nitrite level in the meat to which nitrosylmyoglobin had been added.

Meyer (1981) investigated the effect of nitrite and nitric oxide as inhibitors of the nitrogenase enzyme system of *Clostridium pasteurianum*. The enzyme system consists of two proteins, one of which is an iron protein containing nonheme iron and sulfide as a cubic Fe₄S₄ cluster. Nitric oxide was found to rapidly and irreversibly inactivate the iron protein. The results suggested that nitrite is converted to nitric oxide, which then disrupts the iron-sulfur cluster of the protein and thereby inhibits nitrogenase activity.

Woods et al. (1981) found that the addition of nitrite to a suspension of *C. sporogenes* in a glucose medium caused a rapid decrease in intracellular ATP concentration and an accumulation of pyruvate in the medium. The accumulation of pyruvate was the result of inhibition of the phosphoroclastic oxidoreductase. This enzyme consists of a single protein molecule containing thiamine pyrophosphate and a single nonheme chromophore. Nitrite did not react directly with ferredoxin, but there was decreased activity in the ferredoxin from cells preincubated with nitrite. The authors concluded that, although nitric oxide can react with ferredoxin *in vitro*, the main site of inhibition within intact cells is the reaction of nitric oxide with the enzyme pyruvate-ferredoxin oxidoreductase. Woods and Wood (1982) next reported that adding sodium nitrite to cell suspensions of *C. botulinum* led to an accumulation of pyruvic acid, suggesting that nitrite inhibits the phosphoroclastic system.

Reddy et al. (1983) used electron spin resonance to examine the spectra of botulinal cells treated with nitrite and ascorbate. They concluded that botulinal cells contain iron-sulfur proteins that react with nitrite to form iron-nitric oxide complexes, with resultant destruction of the iron-sulfur cluster. With addition of sodium ascorbate the intensity of the reaction was stronger, thereby verifying observations that ascorbate increases the antibotulinal efficacy of nitrite in meat products. Their results supported the theory that nitrite is inhibitory to *C. botulinum* by inactivating iron-sulfur enzymes (e.g., ferredoxin) that are essential for growth.

Vahabzadeh et al. (1983) found carbon monoxide to be ineffective as a replacement of nitrite for inhibition of *C. botulinum* in pork. Nitric oxide was inhibitory, but this could be attributed to the formation of analytically detectable nitrite in the meat. Adding ferric chloride or myoglobin decreased the antibotulinal efficacy of nitrite. Adding EDTA or denatured nitrosylated myoglobin increased the efficacy of nitrite. The decreased inhibition by nitrite when iron compounds were added appeared to be the result of a reduction in residual nitrite levels in the product rather than to the iron serving as nutrient to stimulate botulinal growth. They concluded that the antibotulinal effect of nitrite and related compounds is probably the result of inhibition of the iron-containing enzymes within the botulinal cell rather than to the complexing of iron, a nutritional requirement for growth.

The debate over whether clostridial ferredoxin or pyruvate-ferredoxin oxidoreductase is the site of nitrite inhibition for *C. botulinum* was again addressed by Carpenter et al. (1987). Their results led to the conclusion that both are inactivated. On the basis of the research conducted by these researchers and others, it is fairly certain that the mechanism of nitrite involves the inactivation of iron-sulfur proteins, such as ferredoxin and pyruvate oxidoreductase. It appears that the hypothesis proposed earlier is fairly close (Tompkin et al., 1978). One aspect that the more recent data suggests is that the interaction of nitric oxide with iron-sulfur complexes is irreversible.

Research by Payne et al. (1990a,b) examined the effect of nitrite and iron-sulfur-nitrosyl complexes on iron-sulfur clusters in *C. sporogenes* cells and isolated proteins. They were unable to demonstrate a direct action of the compounds on the iron-sulfur proteins, leading them to doubt that the inhibitory effect of nitrite is on preformed iron-sulfur proteins of the bacterial cell. After continued research on the mechanism of compounds associated with the Perigo factor, the efficacy of sodium nitroprusside was explored (Joannou et al., 1998). Sodium nitroprusside was found to be a very effective inhibitor of *C. sporogenes*, comparable to the inhibitory properties of the anion of Roussin's black salt. Nitroprusside was used as a model to investigate the inhibitory mechanism of sodium nitroprusside and, more generally, the toxicity of nitrosyl compounds to bacteria. The cell-wall surface was the primary point of attack for the nitrosyl complex associated with nitroprusside. The cell walls showed a 90% decrease in thiol content when compared with untreated cells. In addition, blisters at the surface of the cells were observed when viewed under the electron microscope. In the research of Duncan and Foster (1968b) such blister formation was not reported following exposure to sodium nitrite. Additional information and views on the mechanism of nitrite are available in Roberts et al. (1991) and Roberts and Dainty (1991).

Miscellaneous Studies in Laboratory Media

Botha and Holzapfel (1987) tested the effect of nitrite on *Sporolactobacillus* isolates from a variety of sources. In a broth medium at pH 6.0, 30% to 50% less growth was observed after 5 days at 35°C with 100 or 200 µg/ml of sodium nitrite compared with no added nitrite.

Gibson and Roberts (1986b) demonstrated the interactions of pH, incubation temperature, sodium chloride, and sodium nitrite on the inhibition of *C. perfringens* and fecal streptococci in broth media. The fecal streptococci proved to be quite resistant to nitrite. A high salt concentration and low incubation temperatures were the more important factors influencing the inhibition of these bacteria. The inhibitory effect of nitrite against *C. perfringens* was enhanced with a decrease in pH, a decrease in temperature of incubation, and/or an increase in the salt concentration. The relative sensitivity of *C. perfringens* to 3% to 4% salt could explain why there have been few outbreaks in *C. perfringens* foodborne illness involving cured meats. Most cured meats have traditionally had brine levels of 3% or greater.

The effect of nitrite on the growth of *Listeria monocytogenes* was examined in trypticase soy broth by Shahamat et al. (1980). Sodium nitrite was added to the broth before autoclaving. At the pH values (6.5, 5.5, and 5.0) found in cured meats, some reduction in growth was observed with the addition of 200 µg/g of sodium nitrite. However, the data more strongly demonstrate that *L. monocytogenes* is quite resistant to the inhibitory effect of nitrite, although the nitrite had been autoclaved with the broth.

Juntilla et al. (1989) found the rate of death of *L. monocytogenes* to be similar in fermented sausage manufactured with different levels of salt (3.0% and 3.5%) and sodium nitrite (50, 120, and 200 µg/g). The final pH of the products was about pH 4.6. It can be concluded that factors (e.g., decreasing pH and a_w) influenced the rate of death.

Buchanan et al. (1989) tested the effect of nitrite on *L. monocytogenes* in a broth medium at pH 7.5 and 6.0. The incubation temperature (5°C, 19°C, 28°C, and 37°C) had the strongest effect on growth rate and lag-phase duration. Consistent with previous reports over the past 60 years, nitrite was not an effective inhibitor at pH 7.5. At pH 6.0, which is more typical of cured meat and

poultry products, nitrite was inhibitory. When comparing 0, 50, 100, and 200 $\mu\text{g/g}$ of sodium nitrite, the degree of inhibition was most strongly enhanced by reducing the incubation temperature, to a lesser extent by anaerobiosis, and even less by increasing the salt level (0.5% and 4.5%). The results of Glass and Doyle (1989) and Schmidt and Kaya (1990) indicate that the presence of nitrite is of questionable value in determining whether *L. monocytogenes* will grow in processed meat and poultry products at refrigeration temperature.

Spoilage of Cured Meats

Zeuthen (1980) studied the effect of meat pH on the rate of microbial growth on sliced ham. Fresh pork was divided into two groups: low-pH meat (5.5 to 5.7) and high-pH meat (6.3 to 6.5). The hams were boned and pumped with a pickle solution containing sodium tripolyphosphate. The meat was then used for processing as canned hams. After cooking and chilling, the canned hams were opened, sliced, vacuum packaged, and placed at 5°C. The lower pH meat resulted in a ham with a pH of about 6.0 and a residual nitrite level of 38 $\mu\text{g/g}$ after processing. The higher pH meat resulted in a ham with a pH of 6.35 and a residual nitrite level of 90 $\mu\text{g/g}$. Both products had a brine level of approximately 4.0%. During the 7 to 8 weeks of storage at 5°C, the rate of microbial growth was considerably slower in the sliced ham prepared from the lower pH meat.

Collins-Thompson and Lopez (1981) demonstrated that nitrite depletion rate can be increased by certain lactic acid bacteria that are typically responsible for the spoilage of many cured meats. The loss of residual nitrite in bologna as a result of the action of the lactic acid bacteria was estimated as 30%. The authors questioned the role of nitrite during cured meat spoilage because there is evidence demonstrating that nitrite can either limit or enhance the population of lactics.

Competitive inhibition can be a factor affecting the spoilage flora of cured meats. Collins-Thompson and Lopez (1982) reported the inhibition to be species dependent. The growth of *Brochothrix thermosphacta* was restricted when grown on sliced vacuum-packaged bologna with *Lactobacillus brevis* or *L. plantarum* but not with *L. viridescens* or *Leuconostoc mesenteroides*. Inhibitory species caused the pH of the bologna to decrease to 5.3 within 2 weeks at 5°C. Noninhibitory strains decreased the pH of the bologna to only 5.5 within 4 weeks. The authors suggested that inhibitory strains produced specific antibiotic substances other than hydrogen peroxide or lactic acid. The data of Grau (1980), however, demonstrate that inhibition can be dependent on the concentration of undissociated lactic acid and the degree of anaerobiosis existing at the site of growth.

Under certain circumstances the level of analytically detectable residual nitrite can influence microbial inhibition. Thus, the rate of nitrite depletion in cured meats can be important. Collins-Thompson and Lopez (1982) and Dodds and Collins-Thompson (1984) further demonstrated that lactic acid bacteria, particularly homofermentative lactobacilli, can accelerate nitrite depletion. *L. viridescens* was among the most active species. The depletion of nitrite was attributed to enzymatic activity (i.e., nitrite reductase) rather than lowering the pH of the medium. Subsequent tests demonstrated that a strain of *L. lactis* was able to reduce nitrite to N_2O (Dodds and Collins-Thompson, 1985).

Collins-Thompson and Thomson (1986) found two strains of *L. plantarum*, commonly involved in the spoilage of cured meats, that were resistant to the addition of 50 $\mu\text{g/g}$ of sodium nitrite if grown aerobically in APT broth. Both cultures were sensitive to the inhibitory effect of nitrite under anaerobic but not under aerobic conditions. When grown anaerobically, both strains accumulated manganese in their cells. The significance of these observations was discussed, and some interesting questions were posed. For example, it was suggested that in trace metal metabolism, nitrite may stimulate the uptake and transport of ions (e.g., manganese).

By 1988 it was established that nitrite reduction can occur in some strains of *L. plantarum*, *L. acidophilus*, *L. lactis*, *L. viridescens*, *L. leichmannii*, and *L. buchneri* (Wolf and Hammes, 1988). The nitrite reductases generally are heme-containing enzymes; however, the lactic acid bacteria

were considered incapable of synthesizing heme compounds. Following the clue provided by Whittenbury (1964) that some strains of lactobacilli produce heme-containing catalase when grown in the presence of blood or hematin, Wolf and Hammes (1988) found all the strains of *L. plantarum* tested were able to reduce nitrite when hematin was provided to cell suspensions. In addition, all the strains of *L. plantarum* and *L. sake* tested were able to produce catalase when provided with hematin. Protoporphyrin, an iron-free compound, did not activate nitrite reductase or catalase activity, whereas other iron-containing porphyrins (i.e., hemoglobin, myoglobin, hematin) did activate the enzymes.

Nielsen (1983a) studied the effect of sodium nitrite (0, 100, and 200 µg/g) on the spoilage flora of a sliced vacuum-packed bologna-type sausage. *B. thermosphacta*, Enterobacteriaceae, and *Moraxella* and *Moraxella*-like organisms were increasingly inhibited by increasing the nitrite concentration and/or decreasing the temperature of storage (2°C, 5°C, 10°C, and 20°C). Gram-positive cocci, yeasts, and lactic acid bacteria were inhibited to a limited extent. This led to the lactic acid bacteria becoming the dominant spoilage flora in the vacuum-packed product. Gram-negative bacteria often constituted the major flora of the product without nitrite. Also, as the storage temperature increased, the Gram-negative flora proliferated in product with nitrite.

Nielsen (1983b) subsequently studied the effect of sodium nitrite (0, 100, and 200 µg/g) on the spoilage flora of cooked pork loin. The formulated raw meat was stuffed into a casing, cooked, chilled, sliced, and then vacuum packed. Nitrite inhibited the growth of *B. thermosphacta* and Enterobacteriaceae at all temperatures of storage (2°C, 5°C, and 10°C). Again, the lactic acid bacteria, being insensitive to the presence of nitrite, dominated the spoilage flora in product with nitrite. It was concluded that nitrite has a significant effect in extending the keeping quality of vacuum-packed cured meat when used in combination with low storage temperatures and/or the use of packaging films with a low oxygen permeability.

Silla and Simonsen (1985) studied the rate and type of spoilage of four cured meat products packaged under vacuum and in modified atmospheres. Lactobacilli were the dominant flora in all four products at the time of spoilage. High levels of *B. thermosphacta* may also have significantly influenced spoilage of two of the products. A correlation was found between the nitrite concentration at the time of slicing and packaging and the time for spoilage to occur.

Chyr et al. (1980) found the most important factors influencing the rate of microbial spoilage of liver sausage to be the degree of cooking and the level of nitrite. A marginal thermal process (i.e., 63°C to 68°C) led to the growth of enterococci during storage at 7°C. Increasing the level of sodium nitrite (0, 50, 100, and 150 µg/g) retarded microbial growth. Omitting nitrite resulted in a perfume-like odor that was associated with the growth of enterococci. The level of surviving enterococci decreased as the processing temperature increased (63°C, 68°C, and 74°C). Using good quality raw materials, 156 µg/g of sodium nitrite, and processing to 68°C resulted in a shelf life of more than 16 weeks at 5°C. The spoilage of liver sausage was shown to be the result of *S. faecalis* (Chyr et al., 1981). The spoilage flora of the liver sausage prepared without nitrite also included *Pediococcus pentosaceus*. The *Pediococcus* isolate was not as heat resistant as the enterococcus. The enterococcus survived 65°C for 5 minutes or 60°C for 60 minutes with only 10³ cells/ml broth. An industry survey found that liver sausage was commonly cooked to an internal temperature of 65°C.

Asplund et al. (1988) reported that the growth of *B. cereus* in liver sausage has been a problem in Finland, particularly in the summer. If the product is temperature abused, *B. cereus* can grow in the product even with the normal curing ingredients of salt, phosphate, and sodium nitrite (120 µg/g). Additional additives were tested to reduce this risk. The growth of *B. cereus* was delayed or prevented by including a combination of erythorbate, gluconolactone (glucono delta lactone, GDL), and citric acid in the product. Adding GDL was considered the most important. Product with GDL had a pH value of 5.7 to 5.8, about 0.2 to 0.3 units lower than with the other variables tested.

Kafel and Jozwik (1987) investigated the spoilage rates of commercially produced products from 6 meat processing plants in Poland. Polish regulations require the heating of perishable canned

meats to a minimum internal temperature of 68.8°C. The regulations also require that product from each “lot” be incubated at 37°C for 3 days to assess the relative stability and quality of the product. From among 4322 cans of 8 different classes of perishable canned cured meats, 980 cans, or 23%, swelled. When spoilage first occurred in each lot, additional cans (8290) were removed from storage at around 8°C and incubated at 37°C for 3 days. The time elapsed was normally 7 to 10 days after production. The spoilage rate of this second group of cans was 4%. The authors assumed that the lower spoilage rate was the result of the death of spores that had been heat injured and then subsequently exposed to the effect of salt and nitrite. These data reflect the influence of a mixture, of naturally occurring spores in fairly large cans of commercial product.

The effect of nitrite on the aerobic spoilage flora of raw chicken patties was studied by Bushway et al. (1982). The ground meat was aerobically packaged and stored at 4°C to 5°C. Nitrite was considerably more effective in white meat than in dark meat. With the addition of sodium chloride (2.5%), even a low level of nitrite (40 µg/g) caused some inhibition in white meat but not in dark meat.

Bushway and Jen (1984) found lower residual nitrite levels in chicken white meat than in dark meat, whether cooked or raw. This could be influenced by the fact that white meat is lower in pH and iron content than dark meat. Bushway and Serreze (1984) found that adding iron to raw white meat caused an increase in the rate of aerobic microbial growth. If nitrite (130 µg/g) was also added, the effect of iron on aerobic microbial growth depended on the amount of iron added. Low levels of iron (5 to 15 µg/g) reduced the efficacy of nitrite. However, high levels of iron (40 to 160 µg/g) increased the efficacy of nitrite. The reason for this effect on the aerobic spoilage flora was not known.

Bushway and Serreze (1984) also studied the effect of adding EDTA to raw dark chicken meat. Adding EDTA alone had no significant impact on the rate of aerobic microbial growth. Adding EDTA enhanced the efficacy of nitrite and caused an increase in the lag phase for aerobic microbial growth. Perhaps some adjustment occurred in the microbial population. The authors also investigated the effect of the inherent pH of the meat on the efficacy of nitrite. The pH of the white and dark meat was 5.6 and 6.4, respectively. The antimicrobial effect of nitrite was blocked when the pH of the white meat was adjusted to 6.4. Likewise, the efficacy of nitrite increased when the pH of dark meat was adjusted to 5.6. This led to the conclusion that the inherent pH of the meat is the major factor determining the effectiveness of nitrite on the rate of aerobic spoilage in chicken meat. The authors concluded that the differences in iron content between the dark and white meat may contribute in a small way to the differences observed. A test was not conducted in which the pH of the two meats was adjusted and iron was added along with the nitrite.

SUMMARY FOR 1980–1990

1. The NAS concluded that the evidence available about nitrite does not indicate that nitrite can act directly as a carcinogen in animals. Furthermore, the NAS was not able to conclude that nitrate can act as either a carcinogen or mutagen. These conclusions meant that neither nitrite nor nitrate would be banned as food additives.
2. A means to quantitatively compare the data from various challenge tests based on the probability of outgrowth and toxigenesis from one spore was proposed. This provided a basis for assessing the risk associated with different cured meats.
3. Tests demonstrated that nonproteolytic, psychrotrophic strains of *C. botulinum* can multiply and produce toxin in dry cured hams during the early stages before the salt and nitrite levels penetrate and reach inhibitory levels. This could explain the high number of cases that have occurred during certain periods in Europe (Tompkin, 1980).
4. Extensive studies in meat and meat slurries demonstrated the interactions of nitrite, pH, isoascorbate, phosphate, and thermal process on botulinal outgrowth. This led to the development of a model system for predicting botulinal outgrowth in food, a concept

that has been expanded to include other pathogens and has gained acceptance throughout the world.

5. Some research continued on the Perigo factor, but no evidence was generated to link the Perigo factor to the antimicrobial effect that has been demonstrated in perishable, cured products that receive no heat treatment or are cooked to final internal temperatures in the range of 60°C to 75°C.
6. Alternatives to nitrite continued to be investigated, but none have been adopted commercially.
7. The USDA adopted a regulation for bacon that requires a maximum of 120 µg/g sodium nitrite and the addition of 550 µg/g sodium ascorbate or isoascorbate. Other available options have not received commercial acceptance.
8. The mechanism of nitrite inhibition differs in different bacterial species.

SINCE 1990

C. perfringens has not been a concern in cured meat and poultry products as evidenced by the relative lack of research on this pathogen during the past 40 years. This probably reflects the effect of the higher salt levels and nitrite found in cured products compared with noncured products. Despite this evidence, the USDA in 1988 initiated a series of increasingly restrictive policies on the rate of chilling for perishable cured meat and poultry products manufactured under USDA inspection. This is a case where the epidemiologic data indicate a negligible public health concern for cured meats but the evidence from challenge studies and predictive modeling suggests otherwise. This situation is a reminder of Morris Ingram's frustration with the increase in research on nitrite's role in botulinal inhibition in the 1970s. At the time he stated, "What we need at the present time, in my opinion, is not more inoculated pack experiments but a rationale for interpreting them" (Ingram, 1974). The debate over the risk of *C. perfringens* in commercially manufactured cured meat and poultry products has continued into the new millennium and serves as an example for the need of a risk assessment to clarify appropriate risk management policies.

The potential impact of various blood fractions as ingredients in sausage was investigated (Miller and Menichello, 1991). A beef sausage formulated with 156 µg/g sodium nitrite and an average iron level of 20 ± 5 µg/g was used as the control. Sausage formulated without sodium nitrite became toxic within 1 week compared with 3 weeks when nitrite was present. Adding hemoglobin, red blood cells, or whole blood reduced the time for botulinal outgrowth and toxin formation compared with the control with nitrite. Adding dried plasma, however, delayed the time for toxin to be detected. In subsequent tests the effect of various decolorized blood fractions was evaluated. Again, the rate of toxin formation was inversely related to the level of iron, and the addition of plasma delayed the time for toxigenesis compared to the control sausage (Miller et al., 1993).

Since 1990 there has been increased interest in control of *L. monocytogenes* in ready-to-eat foods. Buchanan and Phillips (1990) studied the growth of *L. monocytogenes* in tryptose phosphate buffer under a wide variety of conditions (salt, pH, temperature, aerobic/anaerobic conditions) and found sodium nitrite to interact with combinations of the factors tested. The database was incorporated into the USDA Pathogen Modeling Program.

McClure et al. (1991), using an automated turbidimetric system with multiwelled plates containing trypticase soy broth, found the efficacy of sodium nitrite was temperature and pH dependent. At 20°C and below no visible growth occurred even with 50 µg/g sodium nitrite and a pH of 5.3. At pH values of 6.0 sodium nitrite had little effect in delaying the time to detect visible growth except at the highest level tested (200 ppm) and a temperature of 15°C or below. At pH 6.0 and 5°C no growth was observed with any of the levels of sodium nitrite evaluated (50, 100, 200, 400 µg/g). In the absence of nitrite, very slow growth did occur. Autoclaving the sodium nitrite in the medium did not increase the degree of inhibition against *L. monocytogenes* to the degree that

had been observed with clostridia (see Perigo Factor). McClure et al. (1996) subsequently investigated the effects of salt, pH, temperature, and sodium nitrite in laboratory medium and a predictive model for the growth of *L. monocytogenes*.

An extensive series of experiments led to the conclusion that nonthermal inactivation of *L. monocytogenes* by sodium nitrite is pH dependent and related to the concentration of undissociated nitrous acid (Buchanan and Golden, 1995; Buchanan et al., 1997).

Duffy et al. (1994) inoculated a variety of vacuum-packaged cooked sliced meats with *L. monocytogenes* and found the lag time increased and the rate of growth decreased at 0°C and 5°C with the addition of sodium nitrite (0 to 315 µg/g). Three µM of residual undissociated nitrite doubled the time taken for a 3 log₁₀ increase in numbers. As in the case of earlier research with *C. botulinum*, the effectiveness of sodium nitrite was significantly increased by the addition of sodium ascorbate.

Farber et al. (1995) examined the effect of sodium nitrite (0 and 200 ppm), salt (1%, 3%), sodium erythorbate (0, 550 µg/g), and temperature of storage (4°C, 10°C) on the rate of growth of *L. monocytogenes* in liver pâté. Only temperature proved to be a significant factor. Perhaps the high iron content of liver pâté negated the effect of sodium nitrite and sodium erythorbate in this product, as had been reported earlier for *C. botulinum*. Liver paté has been implicated in outbreaks of listeriosis in the United Kingdom, Australia, and the United States.

Qvist and Bernbom (2000) manufactured bologna-style sausage with 60 and 150 µg/g sodium nitrite and found neither level retarded the growth of *L. monocytogenes* in the sliced vacuum-packaged product when stored at 4°C or 8°C.

Farkas and Andrassy (1992a) described a test system to evaluate the combined effects of formulation, heat, inoculum level, and other factors for inactivating spores and preventing the outgrowth of survivors. Using *C. sporogenes* (9 × 10⁵/ml) in reinforced clostridial medium, they found that broth adjusted to pH 5.8 and containing 3.5% sodium chloride and 50 or 200 µg/g sodium nitrite inhibited growth during 125 days storage at 37°C. If the broth had been adjusted to pH 6.7, a combination of heat and sodium nitrite was necessary to prevent survival and growth (F₀ = 0.2 with 200 µg/g, F₀ = 0.7 with 50 µg/g). Applying the procedures described by Hauschild (1982), they estimated the various formulations and thermal processes provided the equivalent of 4 to 7 log₁₀ destruction of *C. sporogenes*. The model test system was then used to evaluate various combinations of heat, irradiation, and additives to achieve shelf stability of canned luncheon meat (Farkas and Andrassy, 1992b), although sodium nitrite concentration was not varied. The procedures described show promise for estimating and comparing the combined effects of multiple treatments on pathogen survival and growth.

The effect of salt and nitrite on the growth of 24 lactics isolated from vacuum-packed cooked sausage was evaluated by Korkeala et al. (1992). Low levels of salt (1% to 2%) enhanced growth, but levels above 3% were inhibitory. *Leuconostoc* isolates seemed more sensitive to the effects of sodium nitrite and salt in comparison to the homofermentative lactobacilli. Sameshima et al. (1997) examined the effect of nitrite on three lactics in vacuum-packed cooked sausage made with and without nitrite. Adding 200 µg/g sodium nitrite to the sausage increased the time required to multiply from 10 cfu/g to 10⁵ cfu/g by 9, 4, and 15 days for *L. viridescens*, *L. mesenteroides*, and *Enterococcus faecalis*, respectively.

Fermented Meats and Dry Cured Hams

Although removed from most cured meat products in the United States during the 1970s, nitrate continued to be used in the manufacture of fermented meats and dry cured hams throughout much of Europe and to a lesser degree in the United States. In regions of Northern Europe, sausages made with nitrite and having pH 5.0 and a sour, smoked flavor can be found. These sausages are produced at temperatures below those generally used in the United States but higher than the temperatures used to manufacture traditional raw fermented dry sausage. Since about 1990, some

TABLE 6.2
Basics of Raw Fermented Sausage Processing in Europe

Processing Characteristics:	High-Acid Sausage	Low-Acid Sausage
Fermentation	22°C –25°C	<12°C
Drying/aging	15°C –18°C	14°C –16°C
Final pH	<5.3	~ 6.0
Added carbohydrate	0.3°C –0.7%	None
Added nitrate/nitrite	Nitrite and, perhaps, nitrate	Nitrate
Added starter culture(s)	Yes	Not necessary
Product characteristics:	Salty, strongly acidic flavor; softer, rubbery or spreadable texture	Salty, round, rich, nonacidic flavor; firm texture

Source: Incze (1992).

manufacturers in the Mediterranean countries have been shifting away from the more traditional, low-temperature processing that involves long holding times and reduction of nitrate by micrococci or staphylococci to yield nitrite and other nitrogen oxides that are necessary for cure color development. The cocci also play an important role in flavor and aroma development. In France, Italy, Spain, Hungary, and the Balkan countries, these traditional raw fermented sausages have had higher pH values (e.g., >5.8) and flavors that are more balanced, complex, and nonacidic. The trend toward using starter cultures and slightly higher fermentation temperatures to reduce processing times is accompanied by certain negative features. Specifically, the rapid production of acid renders the cocci inactive and, thus, incapable of reducing nitrate or influencing flavor development. In Spain, loss of the traditional flavor and increased acidity has led to a decrease in consumer acceptance and declining sales. Several reviews, published since 1987, describe the procedures used and changes occurring in Europe (Incze, 1987, 1989, and 1992; Nagy et al., 1989; Nychas and Arkoudelos, 1990; Lücke, 1994; Flores and Bermell, 1996; Flores, 1997; Fernandez et al., 2001). It is curious that very little information has been published during that period from researchers in North America. The basic parameters of raw fermented sausage processing in Europe are summarized from Incze (1992) in Table 6.2.

A few of the studies pertaining to the manufacture of fermented meats will be summarized. In pursuit of more effective starter cultures for use in manufacturing dry sausage, Wolf et al. (1990) conducted further investigations on nitrite reductase activity among the lactic acid bacteria. Tests on 70 strains of lactic acid bacteria using cell suspensions incubated anaerobically led to two groups. One group, consisting of *L. plantarum*, *L. pentosus*, and *Pediococcus pentosaceus*, was heme-dependent for nitrite reduction and yielded ammonia as the sole product. The other group was able to reduce nitrite in the absence of heme and yielded nitric oxide and N₂O. Subsequent research found the lactic acid bacteria could be separated into heme-dependent and nonheme-dependent catalase producers (Wolf et al., 1991). Tests by Mares et al. (1994) found that sodium nitrite (80 µg/g) reduced the catalase activity of a strain of *L. plantarum* but not *P. acidilactici* or *L. pentosus*. Scannell et al. (2001) found the growth of two strains of *Lactococcus lactis* and production of the bacteriocin lacticin 3147 was inversely related to the added level of sodium nitrite (20, 50, and 100 µg/g) in salami.

In Europe, nitrate has traditionally been used for dry sausages and hams requiring long ripening times. In these processes micrococci convert the nitrate to nitrite, which is responsible for the reddening and other effects. In more modern processes, the rate of fermentation is faster and micrococci become inactive as the result of an accumulation of acid. In studies involving faster fermentation, more than 50% of the initial nitrite disappearance was attributed to conversion to

nitrate. Color stability was mainly affected by the presence of residual added ascorbate (Alley et al., 1992).

Neubauer and Götz (1996) summarized nitrate utilization as being either assimilation or respiration. In assimilation, which may occur aerobically or anaerobically, nitrate is reduced to ammonia that is then incorporated into the cell and used as a source of nitrogen. In respiration, nitrate is used as an alternative electron acceptor when oxygen is not present. Two main forms have been described for respiration and, in both, nitrate reduction is coupled to the generation of a proton motive force that is directly used as a source of energy or transformed into ATP. Members of Enterobacteriaceae reduce nitrate to nitrite, which is then either excreted or further reduced to ammonia. Obligately respiring bacteria (e.g., pseudomonads) reduce nitrate to gaseous oxides of nitric oxide and nitrous oxide, which may be further reduced to nitrogen. Certain bacteria, such as strict aerobes, are able to perform assimilatory nitrate reduction but cannot use nitrate as an electron acceptor. Other bacteria, such as *S. Typhimurium*, do not assimilate nitrogen through nitrate reduction during aerobic growth but grow anaerobically with nitrate as the terminal acceptor. In contrast, certain other bacteria use both assimilatory and respiratory nitrate reduction.

Neubauer and Götz (1996) investigated the nitrate reducing system of *Staphylococcus carnosus*, which is used commercially as starter cultures for fermented sausages and dry cured ham. They found *S. carnosus* reduces nitrate to ammonia in two steps. First, nitrate is taken up, reduced to nitrite, and excreted. Second, after nitrate is depleted the accumulated nitrite is taken up and reduced to ammonia. Synthesis of the nitrate and nitrite reductases is inhibited by oxygen and induced to greater or lesser degrees by nitrate or nitrite, respectively. A study of cell suspensions from seven strains of staphylococci isolated from dry sausage (six) and cheese (one) found all but one (*S. warneri*) was able to synthesize nitrate reductase (Talon et al., 1999). Adding nitrate to the growth medium favored the synthesis of catalase, particularly in *S. carnosus*. *S. warneri* had previously been reported to have no nitrate reductase activity and a low catalase activity and when inoculated into sausage caused the product to become rancid (Berdagué et al., 1993).

A new class of nonfermented raw dried sausages of small diameter that has gained popularity in countries such as Spain and Italy is made with nitrate and/or nitrite, but nitrate alone is more common (Sanz et al., 1998). A study comparing sausages made with only nitrate or nitrite and a 26-day process at temperatures that did not exceed 15°C found the aroma and flavor to be more acceptable when the product was made with nitrate.

Parma and San Daniele hams made in Italy, Karst ham made in Yugoslavia, and Savoie ham made in France involve long ripening times and are made without any added nitrate or nitrite (Leistner, 1986). Morita and coworkers investigated the red pigment found in Parma ham and its formation. Morita et al. (1994) found that *S. carnosus* and *S. caseolyticus* isolated from fresh pork were able to convert metmyoglobin to red myoglobin derivatives. Subsequently, Morita et al. (1996) demonstrated that the red pigment found in Parma ham was much more stable and different from nitrosomyoglobin, the pigment found in hams made with nitrate and/or nitrite. Microbiological examination of 471 isolates from Parma ham found the majority were able to convert metmyoglobin to a red myoglobin derivative. Of the ten strains selected for further study, all were staphylococci (*S. epidermidis*, *S. warneri*, and *S. lentus*). Tests with two strains of *S. lentus* in a sausage mix without nitrite or nitrate found both to produce a pigment in the meat that had the same spectral pattern as the pigment found in Parma ham. Morita et al. (1998) then evaluated a strain of *S. xyloso* and found it to produce a red myoglobin derivative in the same meat system without any added nitrate or nitrite. Spectral analysis and electron spin resonance studies determined the pigment was nitrosomyoglobin, the red pigment that is associated with products made with nitrate or nitrite. The mechanism for the formation of the pigment in the absence of nitrate and nitrite was not determined.

Alternatives to the Use of Nitrate or Nitrite

Concern for the potential toxicologic effects of nitrite and nitrate led to continued research to find an alternative. Among the options considered was the use of a red pigment producing mold used to ferment rice, *Monascus purpureus* (Leistner et al., 1991; Fink-Gremmels et al., 1991; Park et al., 1999; Tseng et al., 2000). In the Far East, particularly in China, rice is fermented with molds of the genus *Monascus* to form a pleasant red color. For more than 1500 years the rice has been dried; ground; and, in this form (called Angkak), added to a variety of foods (e.g., pork, poultry, fish, tofu, rice wine) to improve color, flavor, and keeping quality (Leistner et al., 1991).

Cheese

Nitrate has been added during the manufacture of certain European cheeses for more than 150 years (Pivnick, 1980). Although not permitted in the United States, Canadian regulations permit this practice (Assembly of Life Sciences, 1981). This chapter will not review the extensive literature on the use of nitrate to prevent cheese defects (e.g., late fermentation and gassiness resulting from *Clostridium tyrobutyricum* or *C. butyricum*). An opening to the literature on this subject can be obtained through references cited in Langsrud and Reinbold (1974), Assembly of Life Sciences (1981), and others (Mayenobe et al., 1983; Galesloot and Hassing, 1983; Gilles and Fryer, 1984; Ariga et al., 1984; Gouda and El-Zayat, 1988; Klijn et al., 1995).

Seafood

The comprehensive review by Jarvis (1950) provides very few examples for the use of nitrate (i.e., saltpeter) in the curing of fishery products prior to 1950 throughout the world, and virtually no mention is made of its value as a preservative. Knøchel and Huss (1984b) state that nitrate traditionally has been added to the salt used in producing sugar salted fish in Scandinavia. In Denmark, for example, the allowable level has been 500 mg potassium nitrate per kg fish to provide about 300 µg/g nitrate. They conducted an extensive study in which 0, 25 g, or 50 g of potassium nitrate was added to each of 200 barrels containing 90 to 100 kg fish, 16 kg salt, and 6 kg sucrose (Knøchel and Huss, 1984a,b). The 600 barrels were topped off with brine solution, sealed, and evaluated over 18 months storage at 4°C to 6°C. Adding nitrate caused the herring to be redder in color, but no differences were noted for flavor or texture. As the amount of sodium nitrate increased, fewer barrels were judged to be spoiled with sour, sweet/sour, fruity, or putrid off-odors and off-flavors. The incidence of spoilage was 13, 8, and 2 barrels, respectively, with 0, 25 g, and 50 g of sodium nitrate. The reduction in spoilage was not considered to be the result of microbial inhibition by nitrate or its reaction products (nitrite, nitric oxide, nitrous acid) because a high percentage of the isolates from all three variables were able to reduce nitrate. Nitrate was found to delay the depletion of trimethylamineoxide (TMAO). Nitrate and TMAO serve as alternative electron acceptors in barrel-cured herring and when in sufficient concentration delay growth of the strict anaerobes that are responsible for the most common type of spoilage. The reader may recall that during the 1950s nitrate was found to be an electron acceptor in certain cured meat products.

Two microbial hazards of concern in ready-to-eat fish products are *C. botulinum* and *L. monocytogenes* (Gram, 2001a,b). Cuppett et al. (1987) found that toxin production by *C. botulinum* type E (10^3 spores/g) at 27°C was inhibited for 35 days in smoked whitefish containing 4.4% salt in the water phase. When sodium nitrite (156 µg/g) and sodium ascorbate (550 µg/g) were added, toxin production was inhibited for 56 days. The additives provided no additional protection when the salt level in the fish was reduced to about 2.3%.

Hyytiä et al. (1997) found the addition of sodium nitrite delayed or prevented toxin production by *C. botulinum* type E in vacuum-packed, cold-smoked trout having 3.4% salt in the water phase. In one study with 166 µg/g sodium nitrite, toxin was detected at 4 weeks at 4°C with a high inoculum level (20,000 cfu/kg) and after 4 weeks with a low inoculum (800 cfu/kg). In a second

study with 109 µg/g sodium nitrite and lower inoculum levels (4000 and 140 cfu/kg) toxin was not detected through 6 weeks at 4°C or 8°C; whereas with salt alone, toxin was detected in 3 to 4 weeks. Adding potassium nitrate at 347 or 686 µg/g was equal to or more effective than sodium nitrite. Analysis demonstrated that nitrite was produced during storage. The addition of nitrite or nitrate increased the time the fish was considered acceptable by 3 or 4 weeks at 4°C.

With the availability of lower cost farm-raised salmon the international market for cold smoked salmon has continued to increase. In 1992 more than 32,000 tons shipped internationally from 28 exporting countries to 52 importing countries (Duffes, 1999). In many countries there is no legal requirement for the minimum amount of salt, although a minimum of 3% salt in the water phase is strongly recommended (Huss et al., 1995). A level of 3% salt in the water phase will yield perfectly acceptable, lightly preserved fish products (cold smoked fish, gravad fish, brined shrimp), and with refrigeration this combination is effective in inhibiting growth of *C. botulinum* (Huss, 1997). In France, with a production of 12,380 tons of cold smoked salmon in 1997, the salt in the water phase ranged from 2% to 4.6% (Duffes, 1999). Considering the lack of regulations and apparent variability in salt concentration in large quantities of cold smoked salmon distributed domestically and internationally, it would appear the risk of botulism is lower than the research has suggested.

Two options are available in the event commercially manufactured product is implicated in botulism. First, a minimum concentration for salt in the water phase can be established. Controlling the salt in the water phase and temperature should be adequate, but, if necessary, sodium nitrite could be used as an additional control measure. Until there is evidence of a need, it is highly unlikely that sodium nitrite will be adopted as an additive in smoked fish in Europe. In response to outbreaks of botulism in the United States in the early 1960s when less information was available about commercial practices and from research, federal regulations were adopted requiring vacuum-packed, smoked fish to contain at least 3.5% salt in the water phase or 3.0% salt if combined with 200 µg/g sodium nitrite (Gram, 2001a). If packaged aerobically, a lower salt content is permitted (i.e., 2.5% salt in the water phase); however, questions still remain about “aerobic packaging” and its value for controlling botulinal outgrowth. The addition of sodium nitrite is limited to smoked salmon, sablefish, shad, and chub.

Following a review of the literature, a panel of experts reached the following consensus for its report to the FDA (Gram, 2001a):

- Psychrotrophic *C. botulinum* can be expected to occur in smoked fish, although the numbers, if present, are low.
- Toxin production can be controlled with a combination of a moderate level of salt (3.5% in the water phase) and cold storage (<4.4°C) for at least 4 weeks.

Furthermore, that combination of salt and temperature, allowing for short periods of elevated temperatures up to 10°C, will prevent toxin production in cold smoked fish for several weeks beyond its sensory shelf life when packaged under reduced oxygen conditions.

The panel was reluctant to recommend expanded use of sodium nitrite despite evidence showing that psychrotrophic *C. botulinum* would be further inhibited, particularly in combination with controlled levels of salt (Pelroy et al., 1982; Cuppett et al., 1987; Hyytiä et al., 1997). The panel expressed concern for the potential carcinogenic effects of nitrosamines, but the panel did not consider the more recent risk assessments demonstrating the safety of dietary nitrite (see section on toxicology). NOCs were not detected in two studies in which nitrite had been added to smoked fish (Pelroy et al., 1982; Cuppett et al., 1987).

Surveys conducted before the year 2000 indicate *L. monocytogenes* is common in smoked fish. This may reflect survival or contamination, depending on the conditions of processing. Following its review of the literature the panel of experts concluded that it is not possible to consistently produce cold smoked salmon that is free of *L. monocytogenes* (Gram, 2001b). It is possible, however,

with strict adherence to good manufacturing practices, to produce cold smoked salmon with low levels, often less than 1 cfu/g and at a low prevalence. Such levels would ensure the number does not increase above 100 cfu/g when the product is held at 5°C and eaten within 3 to 4 weeks. Because of the relatively high tolerance of *L. monocytogenes* to salt and the adverse effect on flavor, increasing the salt concentration above the normal range for smoked fish is an unacceptable option for control. Peterson et al. (1993) found the rate of growth to be similar at 5°C for salt concentrations of 3% to 5% in the water phase. In subsequent research, adding 125 µg/g sodium nitrite resulted in a slower rate of growth in vacuum-packed salmon having 3% salt in the water phase (Pelroy et al., 1994a). Sodium lactate (2%, w/w) was much more effective and prevented growth through 50 days at 5°C when used in combination with 3% salt. Pelroy et al. (1994b) found the addition of sodium nitrite (190 to 200 µg/g) to be very effective in slowing the growth of *L. monocytogenes* in salmon containing 3% salt in the water phase. This combination was more effective when a low inoculum level (10 cfu/g) was used as a challenge. Previous studies had found levels of 0.5 to 11.7 cfu/g in positive samples of cold smoked fish from five plants. The initial level of contamination is an important factor influencing growth (Guyer and Jemmi, 1991; Peterson et al., 1993; Pelroy et al., 1994; Rørvik, 2000).

Effect of Nitrite on Enteric Pathogens

Blanche Koelensmid and van Rhee (1974) observed the growth of three of four *Salmonella* strains at 20°C in ham jelly containing 500 µg/g of sodium nitrite and 5.6% salt. At 5°C, nitrite accelerated the death of all four strains.

Meers and Goode (1965) reported the rapid growth of *S. Typhi* in inoculated canned corned beef stored at room temperature and at 37°C. After reaching 10⁷/g, there was a gradual decline; however, viable cells remained even after 6 months of storage. Cans inoculated with *E. coli* remained flat; cans inoculated with *C. perfringens* swelled within 2 days at 37°C. The authors implied that, because *S. Typhi* has a nitrate reductase, it uses the nitrate in the product in place of oxygen as an alternative hydrogen acceptor; however, tests were not performed to show whether the presence of nitrate influenced growth, nor were analytical data presented for the product tested. Kittaka and Greenberg (personal communication) obtained similar results in corned beef inoculated with three different *S. Typhi* strains, including an isolate from the 1964 Aberdeen outbreak involving canned corned beef. Product from two sources, one domestic and one imported, were used for testing. The domestic product contained 4.13% brine, 14 µg/g of sodium nitrite, and 720 µg/g of sodium nitrate at the time of opening and inoculation; the imported product had 4.26% brine, 15 µg/g of sodium nitrite, and 45 µg/g of sodium nitrate. Growth occurred in both products. A lower maximum population (10⁶ versus 10⁸/g) and slower die-off occurred in the imported product. Viable *S. Typhi* remained after 20 months at 27°C to 38°C.

Goepfert and Chung (1970) inoculated the surface of sliced bologna and liver cheese with *S. Typhimurium* or *Salmonella* anatum. In both products, *S. Typhimurium* declined during the first 2 weeks at 5°C and then remained stable through 6 weeks. *S. Typhimurium* grew in both vacuum-packaged products when held at room temperature. *S. Anatum* grew in bologna but not in liver cheese. The growth of *S. Typhimurium* occurred more rapidly and to higher levels when the packages were opened, rewrapped in Saran™, and held at room temperature. Dehydration prevented growth if the packages were opened and left exposed at room temperature. Chemical data were not provided for either luncheon meat. It is reasonable to assume the products were recently produced.

Davidson and Webb (1973) also studied the growth of *S. Typhimurium* in vacuum-packaged luncheon meats. Survival of the inoculum in product held at 7°C varied with the product and inoculum level. Mock chicken supported the best growth at both temperatures tested (24°C and 37°C). Very erratic growth occurred on bologna at 18°C, but a steady increase in numbers occurred at 24°C. Similar results were obtained on ham. The inocula died in wieners at both 18°C and 24°C, although not before some initial growth at 24°C. At the end of storage, the pH of mock chicken

and bologna was about 6.0, that of the ham was near 7.0, and the pH for the wieners was 4.7 to 4.8. No other chemical data were provided.

Leistner et al. (1973) inoculated vacuum-packaged luncheon meat with a mixture of *Salmonella* strains and held the product at 8°C for 22 days. The rate of growth or death was influenced by the sodium nitrite content (0, 50, 75, and 100 µg/g) and the level of inoculum (100 or 100,000/g). A similar response occurred with enteropathogenic *E. coli*, but a mixture of *Enterobacter*, *Klebsiella*, and *Hafnia* strains readily multiplied at all nitrite levels. Fermented sausage (teewurst) was prepared with four sodium nitrite levels (0, 65, 98, and 130 µg/g) and two *Salmonella* inoculum levels (100 and 100,000). Growth occurred in the sausage containing 0 and 65 µg/g of nitrite. Death occurred after 3 days in the sausage with the lower inoculum and 130 µg/g of nitrite. The growth of *Klebsiella* and *Enterobacter* species on vacuum-packaged luncheon meats at 3°C was reported earlier by Hechelmann et al. (1974). Both genera were prevalent in commercially produced luncheon meats and fermented sausages.

The resistance of *Salmonella* to nitrite in culture media was demonstrated by Roberts and Garcia (1973). Neither of two strains of *S. Typhimurium* was inhibited at pH 6.0 by 160 µg/g of sodium nitrite, whether the nitrite was added before or after autoclaving the medium of Perigo et al. (1967). Castellani and Niven (1955) reported *S. Typhimurium* to be resistant to nitrite (2000 to 4000 µg/g) when added as a filter-sterilized solution to a broth medium having a higher pH of 6.6 to 6.7.

The trend in the United Kingdom toward using less salt for processing ham was studied by Akman and Park (1974). A low-brine ham was prepared with 1.8% to 2.2% salt and 60 µg/g of potassium nitrite and a finished brine level of about 2.8%. A high-brine ham had a brine level of about 6%; nitrite values were not given. In the low-brine ham at 22°C, 4 of 5 strains of *Salmonella* grew readily. *Salmonella* Enteritidis was able to grow at 17°C but not at 10°C. In the high-brine ham held at 22°C, viable counts of the 5 strains remained near the inoculum level. Two strains of *S. Enteritidis* were then used to study the effect of nitrite and salt on growth in nutrient broth at 22°C. At a low pH of 5.0, 5% salt, and 100 µg/g of sodium nitrite, growth was reduced by only about 30%. At a high pH of 7.0 and 5% salt, nitrite was without effect. The authors did not state whether the nitrite was added before or after autoclaving the medium. It was concluded that cooked ham is a more likely source of salmonellosis if prepared with a low salt content.

Stiles and Ng (1979) studied ham and chopped ham obtained fresh from two processors. The products were inoculated with five "enteropathogens" (*C. perfringens*, *E. coli*, *S. Typhimurium*, *S. aureus*, and *B. cereus*) and then vacuum packaged. The moisture content of the hams was 70%; that of the chopped hams was 70% and 59%. Although salt and nitrite values were not reported, pH values were followed through storage. The mean pH for the ham during storage decreased to no lower than 5.58. The pH of the chopped ham with 70% and 59% moisture decreased to 5.47 and 5.78, respectively. All the enteropathogens grew in one or more of the products within 24 hours at 30°C. The primary factors influencing growth were the temperature of storage and growth of competitive flora. It is not possible to assess the influence of nitrite from the data, other than to say that fresh, commercially produced sliced ham and chopped ham did support the growth of the pathogens studied, including *Salmonella*, given the proper circumstances.

Stiles et al. (1979) tested the same bacteria on sliced bologna from two processors. Again, no data were provided for brine or nitrite levels, but pH changes were noted. Growth of *C. perfringens* did not occur under any condition. The other four species were able to grow under certain conditions. Aside from temperature of storage, the primary factor influencing growth was product pH. The product from one supplier remained above pH 6.0, whereas the other frequently decreased to below pH 5.5. This probably reflects the quantity of fermentable carbohydrate added to the product and/or the nature of the competitive flora.

Page and Solberg (1980) found *S. Typhimurium* to utilize nitrate, nitrite, and ammonia as the sole nitrogen source in a chemically defined minimal medium. Nitrite and ammonia were assimilated at comparable rates. Under anaerobic conditions, the fastest generation times occurred in

media containing both nitrate and ammonia. *S. Typhimurium* used nitrite as the sole nitrogen source at concentrations as high as 400 µg/g. At concentrations of 500 µg/g and higher, complete inhibition occurred.

Page et al. (1985) identified a nitrite reductase in the cytoplasmic fraction of *S. Typhimurium* when grown anaerobically with nitrite as the sole nitrogen source. The reductase required NADH and was most active at pH 8.0. When grown anaerobically in a glucose-limited minimal medium with peptone and nitrite, *S. Typhimurium* had shorter generation times and increased cell yields compared with nitrite-free cultures. The presence of nitrite had no effect on aerobically grown cultures.

Gibson and Roberts (1986a) examined the interaction of salt (1% to 10% wt/vol), pH (5.6, 6.2, and 6.8), and sodium nitrite (0 to 400 µg/g) in BHI and incubation at 10°C to 35°C on the growth of *Salmonella* and *E. coli*. The results demonstrate that these bacteria are quite resistant to the effect of salt and nitrite at the levels most commonly present in such foods as perishable cured meats and smoked fish. Thomas et al. (1991), using a gradient gel plate system, found *S. Typhimurium* to be relatively resistant to sodium nitrite across the ranges of salt and pH values found in nonfermented and acidulated foods. The nitrite resistance of *E. coli* was also apparent in tests involving two-dimensional gradient plates incubated at 20°C, 27°C, and 37°C (McClure and Roberts, 1987).

Hughes and McDermott (1989) used a procedure similar to that of Gibson and Roberts (1986a,b) to determine the additional effect of eight different phosphates of *E. coli*. The phosphates generally delayed the outgrowth of *E. coli*. When examined after 10 weeks of incubation, the effect of added phosphate was relatively minor, except when the cultures were incubated at the lowest temperature tested (10°C).

Rice and Pierson (1982) tested the effect of sodium nitrite (0, 50, and 156 µg/g) in frankfurters on a mixture of two strains of *Salmonella*. The franks were inoculated on the surface, vacuum packaged, and incubated at 15°C or 27°C for 21 days. Nitrite was only inhibitory with the highest level of nitrite and when the franks were incubated at 15°C.

Collins-Thompson et al. (1982) were able to isolate Gram-negative bacteria from commercial packages of vacuum-packaged bologna only after storage for 7 to 8 weeks at 5°C. Isolates of *Enterobacter* and *Serratia* species were tolerant to nitrite, being able to use nitrite in broth culture under anaerobic conditions at 5°C.

The rate of death of *Salmonella* Senftenberg in fermented sausage was fastest with 200 µg/g of sodium nitrite and next fastest with 100 µg/g of sodium nitrite (Puolanne, 1977). The death rate was slower with potassium nitrate (150 or 300 µg/g) or a combination of nitrate and a low level of nitrite (75 µg/g of potassium nitrate and 50 µg/g of sodium nitrite). He recommended that a starter culture and/or gluconolactone be added to provide optimum safety.

Collins-Thompson et al. (1984) prepared fermented sausages with sodium nitrite (0, 50, 100, 150, and 200 µg/g) and three strains of *Salmonella*. Growth did not occur during fermentation or drying. The presence of nitrite did not influence the survival of salmonellae. Similar results were observed with *S. aureus* and *C. sporogenes*.

Schillinger and Lücke (1989) measured *Salmonella* growth and/or survival in mettwurst, a cured raw spreadable sausage. Factors influencing *Salmonella* growth in mettwurst that was cured and stored at 20°C include the amount of nitrite and salt, the inherent pH of the meat (normal pH 5.6 to 5.8 versus 6.0 to 6.2), the addition of sugar, and the presence of lactic acid-producing bacteria. This research was primarily concerned with preventing the growth of *Salmonella*. None of the variables tested would likely assure the destruction of *Salmonella* in this product category.

S. Typhimurium numbers decreased in BHI broth adjusted to pH 4.8 and held at 24°C independent of sodium nitrite concentration. At pH 5.8, numbers increased during 72 hours in the presence of 0, 50, and 100 µg/g sodium nitrite, but growth was delayed with 200 µg/g (Turanta and Ünlütürk, 1991). In a traditional Turkish dry sausage that is fermented and dried at temperatures

not exceeding 24°C, the death rate of *S. Typhimurium* was not affected by the addition of 200 µg/g sodium nitrite (Turanta and Ünlütürk, 1993).

The data for *Salmonella* can be summarized as follows. *S. Typhimurium* has a nitrate reductase to reduce nitrate to nitrite and can assimilate inorganic nitrogen from nitrate, nitrite, or ammonia under anaerobic conditions. The data indicate that the nitrite content of cured meats can influence the growth and survival of *Salmonella* and *E. coli*. That cured meats have been implicated in outbreaks of salmonellosis supports the available research that *Salmonella* can survive, if not multiply, in a variety of commercially cured products. Preventing salmonellosis from cured meats is best accomplished by means other than relying on the level of nitrite in the product, particularly because salmonellae are destroyed during the manufacturing process for most cured meats. This is not true for the clostridia.

Inhibition of *E. coli* O157:H7 in a broth system containing sodium nitrite was strongly pH dependent (i.e., pH 5.5) and enhanced by lowering the incubation temperature (Buchanan and Bagi, 1994). In tryptic soy broth 4 strains of *E. coli* O157:H7 at 37°C were inhibited by 200 µg/g sodium nitrite at pH 5.0, but even 1 g/L sodium nitrite was ineffective at higher pH values (Tsai and Chou, 1996). In ground pork held at 5°C or 25°C, sodium nitrite at 70 to 150 µg/g did not affect the growth or survival of *E. coli* O157:H7 (Yu and Chou, 1996). The effect of nitrite in Chinese-style sausage during drying at 50°C to 60°C for different time periods (2.5 to 6 hours) was next studied (Yu and Chou, 1997). This sausage is not fermented or cooked and relies on relatively rapid dehydration to achieve microbial stability. They found some increased death of *E. coli* O157:H7 with the addition of curing agents and drying at time–temperature combinations that appear to cause stress of the inoculum. Adding sodium nitrite did not increase death of *E. coli* O157:H7 when lower temperature and/or shorter time combinations (e.g., 55°C for 4 hours) were used. Riordan et al. (1998) found *E. coli* O157:H7 numbers decreased less than 1 log₁₀ in pepperoni formulated with 2.5% salt and 100 µg/g sodium nitrite, fermented to pH 4.8, and then dried for 7 days. In Lebanon bologna, a fermented noncooked beef sausage, the addition of sodium nitrite (0, 78, and 156 µg/g) had no effect on the death rate of *E. coli* O157:H7 (Chikthimmah et al., 2001).

Inhibition of *Shigella flexneri* in broth incubated aerobically was significantly influenced by the combined effect of nitrite concentration, salt level, pH, and temperature. The inhibitory effect of nitrite was much greater at pH 5.5, the lowest pH tested and with increasing salt levels. Inhibition was much greater at 28°C than at 37°C (Zaika et al., 1991). Another study involving anaerobic conditions yielded similar trends (Zaika et al., 1994).

Inhibition of *Yersinia enterocolitica* in broth incubated aerobically was significantly influenced by the combined effect of nitrite concentration, salt level, pH, and temperature (Bhaduri et al., 1994 and 1995). The effect of sodium nitrite (0, 50, 80, 100, and 120 µg/g) on *Y. enterocolitica* 0:3 to grow and survive during the manufacture of raw dry fermented sausage was examined by Asplund et al. (1993). The inoculum decreased from 1.7×10^5 /g to nondetectable levels by 35 days in the sausages formulated with 80 µg/g nitrite. The highest survival involved a starter culture that produced the least amount of acid and yielded a relatively high final pH of 5.5. Raccach and Henningsen (1997) reported sodium nitrite and salt to act synergistically in the reduction of *Y. enterocolitica* when tested in a meat system.

Inhibition of *Aeromonas* in broth has been found to be highly dependent on nitrite concentration, pH, and incubation temperature (Chou and Tsai, 1996; Pin et al., 1996).

Effect of Nitrite on Yeasts and Molds

A wide variety of yeasts and molds have been isolated from commercially cured meats, but there is no published information that nitrite or nitrate inhibits their growth. The lack of information on the significance of nitrite and nitrate stems from the general acceptance that other factors largely determine the potential for yeast and mold spoilage of cured meats. These factors include water activity; the availability of oxygen; the inhibitory effect of smoke; storage temperature; and, where

permitted, the use of preservatives (e.g., sorbate). In addition, yeasts and molds are of little or no public health importance. Except for a limited number of products to which nitrite and nitrate might be added, they are not major causes of spoilage.

The effect of nitrite on aflatoxin production by *Aspergillus parasiticus* in fresh pork sausage was examined by Obioha et al. (1983). During storage at 5°C for 28 days, no aflatoxin production or mold growth occurred. At 26°C and 37°C, the product spoiled before aflatoxin reached detectable levels. Despite this observation, the results in pork sausage plus a test with media led to the conclusion that high levels of nitrite (e.g., 156 to 200 µg/g) initially restricted the profuse growth of mold. As residual nitrite decreased to less inhibitory levels, the remaining nitrite enhanced growth and aflatoxin production.

Green Discoloration in Cured Meats

Niven et al. (1949) described the characteristics of *Lactobacillus* and *Leuconostoc* strains isolated from commercially cured meats with greenish discoloration. All were salt tolerant and capable of growth at low temperature (5°C). Evidence was presented indicating that the discoloration results from a reaction of hydrogen peroxide with the cured meat pigment. Niven et al. (1954) demonstrated that the heterofermentative lactobacilli responsible for greening on the surface of cured meats were more sensitive to heat than those developing in the center of sausages. Increased heat resistance could be acquired by repetitive exposure to thermal processing. The name *L. viridescens* was given to these bacteria (Niven and Evans, 1957). The mechanism of green discoloration was further elucidated by Shank and Lundquist (1963).

Nitrite burn is another form of green discoloration that is caused by a combination of excessive levels of nitrite and reduced pH (Deibel and Evans, 1957). High levels of nitrite can result from the conversion of nitrate to nitrite by micrococci near the periphery of sausage or by *Staphylococcus* species within the sausage (Bacus and Deibel, 1972). Control of these bacteria lies in proper handling of cooked product to be reworked into succeeding lots of meat. These bacteria must not be given the opportunity to develop a population of increased heat resistance whereby the process will no longer be adequate to assure their destruction. The heat resistance of these bacteria has been reported (Niven et al., 1954; Iwata et al., 1965; Gardner, 1967; Vrchlabsky and Leistner, 1971). Unpublished research by Shaparis and Christiansen has shown D values in minced ham to be $D_{63} = 30.5$ minutes, $D_{65.5} = 12.5$ to 15 minutes, $D_{68} = 5.5$ to 6.5 minutes, and $D_{71} = 1$ to 2 minutes (Tompkin, 1986). Additional information on factors affecting green discoloration by *L. viridescens* and other lactics has been developed by Grant and McCurdy (1986) and Grant et al. (1988).

SUMMARY FOR 1990–2002

1. The most significant development during this period was the growing body of data that dietary nitrate and nitrite are not carcinogenic (see Toxicology).
2. Naturally occurring nitrate in vegetables accounts for more than 85% of the dietary intake of nitrate. Conversion of nitrate in the saliva is the major source of nitrite in humans.
3. Endogenously formed nitric oxide and oxygen radicals can play an important role in human health.
4. Nitrite can contribute to the inhibition of *L. monocytogenes* under certain, but not all, circumstances.
5. A system was developed to evaluate the combined effects of formulation, heat, inoculum level, and other factors for controlling mesophilic clostridia.
6. The role of nitrate and nitrite in the manufacture of traditional and more rapidly produced fermented meats was clarified. The more rapidly produced products closely resemble those that have been produced in the United States over the past 30 to 40 years.

7. The cured ham appearance of traditional European dry cured hams manufactured without the addition of nitrate or nitrite was found to be the result of the formation of stable pigments by naturally occurring staphylococci.
8. The use of nitrate or nitrite as additives in cheese and seafood products was clarified.
9. Nitrite is of relatively little value for controlling Gram-negative enteric pathogens in commercially prepared foods.

REGULATIONS GOVERNING THE USE OF NITRITE AND NITRATE

The regulations limiting the use of nitrite and nitrate in cured meats vary widely among countries and have continued to change. The regulations were summarized in two comprehensive surveys (Meester, 1974; Braathen, 1979). The status of nitrite and nitrate in cured meats and cheese in the United Kingdom was reviewed in 1978 (Ministry of Agriculture, Fisheries, and Food, 1978). Germany reduced the permitted level of nitrite in nitrite-containing curing salt by 20% (Leistner, 1981; Gerhardt, 1995). The most recent summary of the regulations in different countries appears in Cassens (1990).

Nitrite and nitrate levels for curing meats in the United States were recommended by a USDA Expert Panel on Nitrites and Nitrosamines (Food Safety and Quality Service, 1978). The current USDA regulations controlling the use of nitrite and nitrate in meat and poultry products are specified in 9 CFR 317.17 and 424.21-23 of the federal regulations (Code of Federal Regulations, 2002a). Procedures for calculating the amount of nitrite and nitrate that can be added to curing solutions and product were specified in the USDA calculation handbook (Food Safety and Inspection Service, 1986). The use of nitrite and nitrate in the processing of fish products is specified in 21 CFR 172.160, 172.170, 172.175, and 172.177 (Code of Federal Regulations, 2002b). With the exception of bacon and smoked chubs, U.S. regulations do not specify minimum nitrite levels for cured products.

A unique feature of the U.S. meat and poultry regulations is that nitrite and nitrate are added on the basis of the uncured meat in the product formulation. Other countries limit the amount of nitrite and nitrate on the basis of the total weight of the product formulation. The net effect of the U.S. regulations is that less nitrite is added to many products than might be assumed with a regulation that permits 156 $\mu\text{g/g}$ of sodium nitrite. Examples of the effect of this regulation on the maximum allowable level of nitrite in several sausage and loaf products are listed in Table 6.3. As the percentage of extenders and other nonmeat ingredients is increased in the products, the level of added nitrite is decreased. Also listed is the actual amount of nitrite that could be added to the total formulation of the products if the permitted level of nitrite was reduced from 156 to 100 $\mu\text{g/g}$, as proposed by the Expert Panel on Nitrites and Nitrosamines (Food Safety and Quality Service, 1978). It is also common to generate a certain amount of rework during the manufacture of sausage products. An example of rework is the round ends of sausages that are trimmed off before slicing the product. This material is normally used as an ingredient at a level of about 5% in the manufacture of succeeding lots of the same product. Because it has already been cured, the weight of this meat is subtracted from the fresh meat portion of the formulation and the amount of nitrite added is reduced accordingly. This further reduces the nitrite level in the product formulation and, depending on the circumstances, leads to lower residual nitrite levels after processing.

ASSAY METHOD

Because the levels of nitrite and nitrate permitted in foods are established by regulation, the method of assay applicable to each country should be used. Within the United States the methods of assay appear in the Official Methods of Analysis of the Association of Official Analytical Chemists

TABLE 6.3
Examples of Sodium Nitrite Levels Added to Cured Meats in the United States

Product	NaNO ₂ (µg/g) Added on Basis of Meat Content	Actual NaNO ₂ (µg/g) in Total Formulation	Actual NaNO ₂ (µg/g) in Total Formulation if Input NaNO ₂ Is Reduced to 100 µg/g in the Meat
Bologna	156	121	78
Bologna with NFDM ^a	156	117	75
Bologna (imitation)	156	105	67
Franks (meat) ^a	156	115	74
Franks (meat) ^a	156	107	69
Franks with NFDM	156	114	73
Liver sausage ^a	156	152	97
Liver sausage ^a	156	140	90
Cured beef loaf	156	143	92
Blood pudding	156	130	83
Pickle and pimento loaf	156	91	58
Olive loaf	156	94	60
Family loaf	78	50	50
Veal loaf	78	47	47

^a NFDM, nonfat dry milk.

Source: Based on formulations in Pearson and Tauber (1974).

(AOAC international, 2002). International agreement has been reached on the specifications and analytical methods for potassium nitrate and sodium nitrite (JECFA, 1995).

TOXICOLOGY

The acceptable daily intake for potassium nitrate and sodium nitrite have been reported to be 0 to 3.7 and 0 to 0.06 mg/kg body weight, respectively (JECFA, 1995). The values are expressed as nitrate and nitrite ions, respectively, and apply to all sources of intake but do not apply to infants younger than age 3 months.

For potassium nitrate, estimates of the lethal dose have ranged from 4 to 30 g (about 70 to 500 mg/kg body weight), whereas 30 to 60 g of sodium nitrate have been given for 2 months as an acidifying diuretic without manifestation of adverse effects. A realistic estimate of a lethal dose for adults is about 20 g nitrate ion or 330 mg nitrate ion/kg body weight (Gangolli et al., 1994).

Two years of rat studies on nitrate showed no evidence of carcinogenicity. Data on reproductive toxicity for nitrate were limited, and it appears that nitrate *per se* is not genotoxic. The no-adverse-effect dose level was estimated to be 2500 mg sodium nitrate/kg body weight/day (Gangolli et al., 1994).

For sodium nitrite, estimates for the lethal dose have ranged from 2 to 9 g (about 33 to 250 mg/kg body weight), with the lower doses applying to infants who are more sensitive to methemoglobinemia, the criterion most commonly used for nitrite toxicity. The normal range of methemoglobin levels is 0.5% to 2.0%, the uppermost level being found in infants and pregnant women. Cyanosis, as evidence of toxicity, occurs at methemoglobin levels in excess of 10%. Using this criterion, estimates for the toxic dose range from 1 to 8.3 mg sodium nitrite/kg body weight (Gangolli et al., 1994).

Concern for the formation of NOCs in foods began with the discovery that adding sodium nitrite to preserve raw herring for fish meal production resulted in malignant liver disease in cattle

and sheep. In 1963 it was proposed that nitrosodimethylamine formed during the herring meal process was the toxic agent. For humans the greatest exposure to NDMA is from tobacco smoke (Koppang, 1998). Investigations for NDMA in foods led to cured meats, especially bacon when prepared and cooked under certain conditions, and beer from exposing the malt to nitrogen oxides. Controls adopted by the food industry over the past 20 years have led to reductions in the risk of exposure to nitrosamines (Lijinsky, 1999). For example, Cassens (1997) reported that residual nitrite levels in cured meats at the retail level in the United States had decreased over the past 2 decades by approximately 80%. In addition, higher residual ascorbate levels (mean value of 209 $\mu\text{g/g}$) in the cured meat products would reduce the risk of nitrosation reaction products being formed.

An extensive review was conducted during the development of two reports by the NAS (Assembly of Life Sciences, 1981, 1982). Those reports provide the most complete assessment of the safety of nitrite as a food additive for that period. Cassens (1995) has provided an update of the nitrite controversy, including a review of epidemiologic studies seeking to establish a link between intake of nitrate and nitrite from food and water and cancer, particularly among young children. Murphy et al. (1998) concluded that the increase in the incidence of brain and nervous system cancer observed among both children and adults between 1970 and 1990 was not consistent with the decrease in the consumption of cured meats and marked reductions in residual nitrite content during that period. Information presented at a Ceres Forum led to the overall conclusion that available information either does not or is inadequate to support a link between dietary intake of nitrite and NOCs and brain and nervous system cancers in children and adults (Ceres Forum, 1997).

Sodium nitrite is now viewed in an entirely different light than during 1970 to 1990 (CAST, 1997). Assessments conducted during the past decade have led to the conclusion that nitrite, *per se*, is not carcinogenic. Several countries that monitor their foods for nitrate, nitrite, and NOCs have concluded that dietary nitrite and NOCs represent a relatively small contribution to the total body burden of these compounds (Cassens, 1995). Even the American Cancer Society has concluded that "nitrites in foods are not a significant cause of cancer among Americans" (American Cancer Society, 1996).

More recently, an extensive review of the literature and testimony from experts led the California Developmental and Reproductive Toxicant Identification Committee to vote on June 2, 2000, not to list sodium nitrite as a developmental toxicant under the state's Proposition 65 law. This was followed by the final report from the National Toxicology Program in May 2001, summarizing studies conducted over a 2-year period on the carcinogenesis and genetic toxicology of sodium nitrite in drinking water. The report concluded that there was no evidence of carcinogenic activity for sodium nitrite in rats and mice exposed to 750, 1500, or 3000 $\mu\text{g/ml}$. There was equivocal evidence of carcinogenic activity in the forestomach of the female mice tested (National Toxicology Program, 2001). A review of the pros and cons of nitrate and nitrite as food additives in light of the information available by 2001 has been provided by Archer (2002).

An assessment of NOCs by Hotchkiss et al. (1992) concluded that there was only indirect evidence that some portion of human cancer risk is related to NOC exposure and that tobacco was the major source. Endogenous formation of NOCs is influenced by dietary intake and a variety of other factors.

Gangolli et al. (1994) conducted a comprehensive risk assessment on nitrate, nitrite, and NOC and determined that vegetables provide more than 85% of the average daily human dietary intake of nitrate. Endogenous synthesis of nitrate is an additional important source of nitrate. A review of animal toxicologic studies, human effects, and epidemiologic surveys led to the conclusion that there is no firm scientific evidence to recommend drastic reductions beyond the average levels of nitrate encountered in vegetables grown following good agricultural practice.

Gangolli et al. (1994) concluded that dietary sources of nitrite and NOCs contribute relatively small amounts to the body burden. The major source of these compounds in the body is derived

through metabolism of ingested nitrate. In humans, the saliva is the major site for formation of nitrite.

Dykhuizen et al. (1996) reported that ingested nitrate is absorbed from the gastrointestinal tract into the bloodstream and concentrated in the saliva, increasing in concentration up to ten times the level found in plasma. Salivary nitrate is converted to nitrite by nitrate-reducing microorganisms in the mouth. Although this source of nitrite would increase the risk of NOC formation when introduced into the acid conditions of the stomach, the acidified nitrite also has antimicrobial activity that coincides with the formation of nitric oxide. Expelled stomach air contains a high concentration of the antimicrobial gaseous nitric oxide that is enhanced by dietary nitrate intake. As might be expected from other information in this chapter, *in vitro* tests demonstrated the bactericidal effect of acidified nitrite against several enteric pathogens (Dykhuizen et al., 1996). Patients with infective gastroenteritis have increased plasma nitrate levels compared with healthy controls (Dykhuizen et al., 1995).

Nitric oxide is produced by three different nitric oxide synthases (Bogdan, 2001; Chen et al., 2002). There is a growing body of evidence showing that endogenously produced nitric oxide is an important component of the natural defense system to infectious disease in humans (Karupiah et al., 2000; Shiloh and Nathan, 2000; Cherayil and Antos, 2001; Bogdan, 2001; Alam et al., 2002). Nitric oxide is involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes, and chronic degenerative diseases (Bogdan, 2001). More recent research indicates that nitric oxide plays many diverse roles in infection and immunity. It is now clear that inducible nitric oxide synthase (iNOS) "is detrimental in some infectious disease processes" and that it helps to counteract excessive immune reactions, protects to some degree against autoimmunity and functions as an intra- and intercellular signaling molecule shaping the immune response. In addition, nNOS and eNOS (neuronal and endothelial NOS) are now known to participate in important immunological processes such as apoptosis, cell adhesion, autoimmunity, and perhaps antimicrobial defense. We have also begun to learn about the possible role of NO in thymic education" (Bogdan, 2001).

Endogenously formed nitric oxide also plays a role in neurotransmission, blood clotting, and blood pressure control (CAST, 1997). The enzyme nitric oxide synthase catalyzes the oxidation of L-arginine and L-citrulline to release nitric oxide that can be converted to nitrite and nitrate and subsequently excreted (CAST, 1997).

Some of the most important effects that nitric oxide exerts in the vascular wall are potentially vasoprotective because these effects maintain important physiologic functions such as vasodilation, anticoagulation, leukocyte adhesion, smooth-muscle proliferation, and antioxidative capacity (Gewaltig and Kojda, 2002). Impairment of endothelium-dependent nitric oxide activity is believed to be the result of increased production of reactive oxygen species, particularly superoxide. This has been found in atherosclerosis, hypertension, diabetes, hypercholesterolemia, heart failure, and cigarette smoking (Gewaltig and Kojda, 2002; Kurowska, 2002). Inhaled nitric oxide therapy shows promise for improving oxygenation and reducing mortality in preterm infants with pulmonary illness (Srisuparp et al., 2002).

Evidence suggests that nitric oxide and oxygen radicals (e.g., superoxide) are generated in response to various infectious diseases (Akaika and Maeda, 2000). Nitric oxide biosynthesis, particularly through action of an inducible nitric oxide synthase, is beneficial in the case of bacterial and protozoal infections. Generation of nitric oxide in response to viral infections such as influenza virus and certain other neurotropic viruses, however, may have an overall detrimental effect on the host. Excessive amounts of nitric oxide produced for long periods allow generation of peroxynitrite, which can cause oxidative tissue injury through oxidation and nitration reactions of various biomolecules. Nitric oxide also appears to adversely affect the host's immune response (Akaika and Maeda, 2000). On the positive side, recent studies demonstrate that nitric oxide is a potent inhibitor of both rhinovirus-induced cytokine production and viral replication, factors considered of importance in asthma (Sanders, 1999). It has been speculated that nitric oxide plays an important antiinflammatory and antiviral role in rhinovirus infections and that nitric oxide donors may

represent a novel therapeutic approach for the treatment of colds and their related complications (Sanders, 1999).

Research over the past 20 years has clarified many aspects of the role of nitric oxide in physiology and pathology. The research has demonstrated that nitric oxide is a double-edged sword mediator in that it can exert beneficial or detrimental effect depending on the physiopathologic context. This has impaired the development of new drugs that would take advantage of the beneficial properties of nitric oxide (Cirino et al., 2002).

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7 Nisin

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CONTENTS

Chemical and Physical Properties	239
Antimicrobial Activity	240
Units of Measurement and Assay.....	240
Antimicrobial Spectrum.....	241
Mode of Action.....	244
Vegetative Cells.....	244
Spores	246
Factors Affecting Antimicrobial Activity	246
Applications	248
Dairy Applications	249
Pasteurized Processed Cheese.....	249
Natural Cheese	250
Pasteurized Milk and Other Dairy Products.....	251
Canned Foods.....	252
Meat Applications	253
Raw Meat	253
Cooked Meat Products	254
Fish and Shellfish Applications	255
Beverages	255
Fruit Juice.....	255
Alcoholic Beverages.....	256
Miscellaneous Applications	256
Crumpets.....	256
Pasteurized Liquid Egg.....	256
Salad Dressings	257
Fresh Pasteurized Chilled Soups.....	257
Soy and Vegetarian Products.....	257
Cooked Potato Products	257
Fresh Pizza	257
Genetics.....	258
Toxicity.....	259
Legal Status.....	259
References	262

Following its discovery in the late 1920s, the bacteriocin nisin has now been in use as a food preservative for almost 50 years (McLintock et al., 1952). Nisin is a low-molecular-weight polypeptide produced by the bacterial dairy starter culture *Lactococcus lactis* subspecies *lactis*. It has a much broader spectrum than most other bacteriocins, being active against a wide range of Gram-positive bacteria. Since this group of organisms includes heat-resistant bacteria, nisin has become a widely used food preservative because it can maintain or even extend the shelf life of heat-treated foods and contribute to their safety. The characterization of the molecule was first conducted by Mattick and Hirsch (1947), who coined its name from the term “N inhibitory substance.” At that time, the producer organism was classified as Lancefield serologic group N *Streptococcus*. When its potential as a food preservative was realized (Hirsch et al., 1951), the first commercial extract of nisin was developed by Aplin & Barrett Ltd. (now part of Danisco) in 1957.

Nisin has been studied by many research groups, both academic and industrial, because of its many positive attributes — broad-spectrum antimicrobial activity, long history of successful usage as a food preservative, excellent safety record — and its designation as a natural food additive. Nisaplin (which was nisin A) is the first commercial extract of nisin, developed between 1962 and 1965.

Apart from numerous research papers on nisin, several reviews have been published, most notably those by Hurst (1981,1983), Delves-Broughton (1990), Ray (1992), Hurst and Hoover (1993), de Vuyst and Vandamme (1994), Delves-Broughton and Gasson (1994), Delves-Broughton et al. (1996), Thomas et al. (2000), and Thomas and Delves-Broughton (2001). Toxicity testing results demonstrating its safety for human consumption were published in 1962 (Frazer et al., 1962; Hara et al. 1962). Nisin was approved for use in food in 1969 by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Committee on Food Additives and was awarded generally recognized as safe (GRAS) status in the United States in 1988 (FDA, 1988). It remains the only bacteriocin allowed in food as an added preservative.

Food safety is a major concern internationally. Increasing mass production and the longer distribution chains operating in many countries have led to an increase in foodborne illnesses with associated fatalities. Consumers do not want high levels of chemical food preservatives in their food and yet they increasingly look for convenient, long-life food that is of high quality but that is not severely processed (Gould, 1992). Nisin represents a safe, natural method of preservation that can be used as part of a combined preservative system to increase the safety and shelf life of food. Nisin-producing lactococci occur naturally in milk; consequently nisin can be present naturally at low levels in both soured milk and cheese. One of the earliest reports about “inhibitory streptococci” concerned milk in which cheese starter organisms failed to develop (Whitehead and Riddet, 1933). Nisin was also shown to be present in farmhouse cheese (Chevalier et al., 1957). Therefore, it is likely that nisin or substances similar to it have been consumed for a significant length of time without apparent ill effects.

There has been misplaced concern expressed in some quarters resulting from a confusion arising from references to nisin as an antibiotic (Hansen, 1993; Hurst, 1981). Although there is no doubt that therapeutic drugs associated with medical usage should not be used in food applications, nisin is not one of these agents. There are clear differences between this bacteriocin and pharmaceutical antibiotics (Cleveland and Tchikindas, 2001). Before its development as a food preservative, there were attempts to use nisin as a medical drug, but these attempts failed because of nisin’s lack of activity against Gram-negative bacteria and its rapid digestion and breakdown within the body. Generally antibiotics are secondary metabolites. Nisin is a primary metabolite produced by a process involving ribosomal transcription and translation. Nisin is not used therapeutically in human or veterinary medicine nor is it used as an animal feed additive or for growth promotion. Various studies have shown that nisin will not contribute to antibiotic drug resistance. For instance, passaging of bacteria in media containing sublethal concentrations of nisin did not alter the sensitivity of the test organisms to therapeutic antibiotics and other chemotherapeutic drugs (Hossack et al., 1983). We recommend that nisin (and other bacteriocins) should be considered as antimicrobial peptides and not referred to as antibiotics.

Regulatory authorities and food producers acknowledge and appreciate that nisin does not hide poor manufacturing practice. Nisin is not effective against Gram-negative bacteria, yeasts, and molds, or microorganisms that should be killed by normal pasteurization treatments and that are only present in such foods if the heat treatment is inadequate or if postprocessing contamination has occurred. The effectiveness of nisin is also dependent on the bacterial load (Scott and Taylor, 1981b). Nisin will be less effective in a processed food that has a high spore count; this would usually be the result of poor-quality ingredients (Gibbs and Hurst, 1964). Apart from the advantages already mentioned, nisin offers the possibility (within safety limits) of energy saving and consequent cost cutting. Thermally injured spores are more sensitive to nisin, and extended shelf life can be achieved with a combination of nisin and a reduced heat treatment.

CHEMICAL AND PHYSICAL PROPERTIES

The polypeptide can be prepared from culture fluids or the cells of the producer organism. Methods for its concentration and purification have been described (Cheeseman and Berridge, 1957; Bailey and Hurst, 1971; Lipinska et al., 1973; Wilimowska-Pelc et al., 1976; Lee and Kim, 1985), and its synthetic production has been accomplished (Fukase et al., 1988). Nisin is available commercially (e.g., Nisaplin[®] produced by Danisco). Gross and Morell (1971) first elucidated the unusual structure of nisin, which is a 34-amino acid polypeptide with a molecular mass of 3510 Daltons. It is classified as a Class I bacteriocin, molecules characterized by their unusual amino acids. Nisin contains the thioether amino acids lanthionine (Ala-S-Ala) and β -methylanthionine (Abu-S-Ala), as well as amino butyric acid (Abu), dehydroalanine (Dha), and dehydrobutyrine (Dhb; also known as β -methyldehydroalanine). The structure of nisin A, with its distinctive five internal ring structures formed by the disulphide bridges, is shown in Figure 7.1. The natural variant nisin Z differs by a substitution of histidine for asparagine at position 27 (Mulders et al., 1991). The structure and synthesis of nisin have been reviewed (de Vuyst and Vandamme, 1994; Kupke and Gotz, 1996).

Nisin has a flexible, three-dimensional structure, which is determined by its internal thioether rings. It can form dimers or even oligomers. It is a cationic molecule because of its three lysines and either one histidine in nisin Z or two in nisin A (de Vuyst and Vandamme, 1994). It does not contain aspartate or glutamate. The net charge of nisin is pH-dependent. It is amphipathic in character; the N-terminal contains several hydrophobic residues, whereas the C-terminal is more hydrophilic. Nisin has been shown to have emulsifying activity in a comparative study with Tween-80 (a common nonionic food emulsifier) and β -casein (a food emulsion stabilizer), but this activity was highly dependent on pH and nisin concentration (Bani-Jaber et al., 2000).

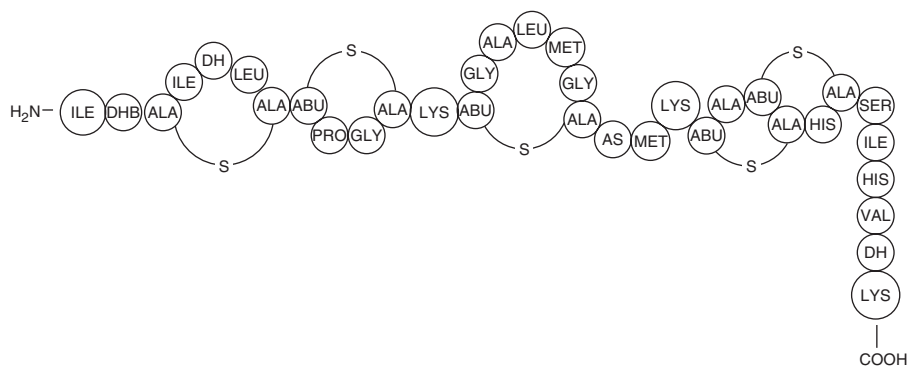


FIGURE 7.1 The structure of nisin. ABU, aminobutyric acid; DHA, dehydroalanine; DHB, dehydrobutyrine (β -methyldehydroalanine); ALA-S-ALA, lanthionine; ABU-S-ALA, β -methylanthionine.

Nisin as a powder is extremely stable if stored at temperatures not exceeding 25°C away from direct sunlight in a sealed container allowing no moisture ingress. Both the solubility and stability of nisin in solution are affected by pH. Optimum heat stability occurs at pH 3 to 3.5 (Davies et al. 1998). This was contrary to previous work by Tramer (1964, 1966). The effect of processing temperature and pH on the stability of nisin in different foods was studied by Heinemann et al. (1965). Their results show that in low-acid foods at pH 6.1 to 6.9, heating for 3 minutes at 250°F (121°C) destroyed 25% to 50% of the added nisin. A similar degree of destruction was reported for highly acid foods having pH values of 3.3 to 4.5. At high pH, Liu and Hansen (1990) suggested that the Dha and Dhb residues become more susceptible to modification as a result of the presence of nucleophiles. Nisin is, however, successfully used in high-pH/heat-treated food products such as canned vegetables and pasteurized liquid egg, suggesting that the molecule is protected to some extent by food components (Delves-Broughton, 1990; Delves-Broughton et al., 1992).

Liu and Hansen (1990) reported nisin solubility at pH 2.2 as approximately 56 mg/ml, dropping to 1.5 mg/ml at pH 6 and 0.25 mg/ml at pH 8.5. Solubility in foods is never a problem because nisin levels are always very low, with levels of usage in food rarely above 0.025 mg/ml (equivalent to 1000 mg/kg Nisaplin®). However, research in Danisco laboratories has shown that care must be taken when preparing high-concentration nisin solutions from commercial preparations such as Nisaplin®. These may be intended as stock solutions for the preparation of test dilutions in small-scale laboratory experiments. It is recommended that such solutions should not exceed a maximum concentration of 250 µg/ml; otherwise a loss of activity may occur as a result of salt precipitation or filtering.

Nisin has no significant taste and imparts no negative taste to foods in which it is used. In triangle taste trials conducted by Danisco, volunteers could not detect 200 mg/L Nisaplin® in mineral water (equivalent to 5 µg pure nisin/g).

ANTIMICROBIAL ACTIVITY

UNITS OF MEASUREMENT AND ASSAY

The activity unit for nisin was first defined as a Reading Unit: the minimum amount of nisin required to inhibit the growth of one cell of *Streptococcus agalactiae* in 1 ml of broth (Tramer and Fowler, 1964). An international nisin reference preparation was later established by the WHO Committee on Biological Standardisation (Anonymous, 1970), and nisin activity was then defined in International Units, equivalent to 1 µg of this preparation. The current term, International Unit, is the same value as the Reading Unit. The specifications for the purity and identity of nisin were set in 1969 by the FAO/WHO Joint Expert Committee on Food Additives (Anonymous, 1969). Pure nisin was deemed too potent for use in food, and a specification was set for commercial nisin concentrates containing at least 2.25% biologically active nisin. The nisin preparation Nisaplin® was developed by Aplin & Barrett (now Danisco) between 1962 and 1965. This has a standard potency of 1 million International Units per g, and contains 2.5% nisin A with the remainder made up of added salts and milk solids derived from the fermentation of a modified milk medium by *L. lactis* subspecies *lactis*.

In this chapter nisin is expressed as levels of pure nisin (µg/ml or µg/g). To convert these units to equivalent Nisaplin® levels (mg/L, mg/kg) or to International (Reading) Units (IU/ml, IU/g), the levels of pure nisin should be multiplied by 40. For example, 1 µg/g pure nisin is equivalent to 40 IU/g or 40 mg Nisaplin®/kg.

The horizontal agar diffusion test, first developed by Tramer and Fowler (1964) and Fowler et al. (1975), is the most widely used nisin assay. The Gram-positive bacterium *Micrococcus luteus* is used as an indicator organism because it is very nisin-sensitive and grows well overnight at 30°C, producing clearly distinguished and measurable zones of inhibition in the presence of nisin. Nisin is extracted from the test food by an acid-heat treatment, and this bioassay method contains

numerous control procedures to ensure nisin activity is not confused with other antimicrobial agents that may be present in the food. There have been several reported improvements to this test (Rogers and Montville, 1991; Joosten and Nunez, 1995; Wolf and Gibbons, 1996). An incorrect measurement of activity would be obtained by assaying nisin Z samples against the nisin A FAO/WHO standard used for calibration in the horizontal agar diffusion assay, because nisin Z diffuses more rapidly than nisin A.

An enzyme-linked immunosorbent assay (ELISA) has been developed using polyclonal anti-serum raised in sheep to nisin A and conjugated to horseradish peroxidase (Falahee et al., 1990; Falahee and Adams, 1992). This latter test does not measure biological activity. Comparative studies of processed cheese assayed by ELISA and the horizontal agar diffusion bioassay showed a lack of correlation (Aplin & Barrett Ltd., unpublished results), which was thought to result from the test measuring a partially degraded nisin molecule that had lost biological activity. This could be corrected by developing an antibody that detected distal parts of the molecule, ensuring that nondegraded nisin only would be measured. An ELISA system using nisin Z polyclonal antibodies in rabbits also suffered from similar problems, again most probably because of detection of inactive degraded nisin or possibly biologically active nisin that was bound within the food (Bouksaim et al., 1999).

A rapid nisin assay method has been described that measures nisin activity by adenosine triphosphate (ATP) release from *Lactobacillus casei* as a result of exposure to nisin, detectable by bioluminescence (Waites and Ogden, 1987). An *L. lactis* construct strain sensitive to nisin has been reported, capable of transducing the signal into detectable bioluminescence (Wahlstrom and Saris, 1999). Rose et al. (1999) described detection of nisin by matrix-assisted laser/desorption time-of-flight spectrometry.

ANTIMICROBIAL SPECTRUM

Nisin has a broad spectrum of activity against Gram-positive bacteria; nisin-sensitive organisms relevant to food are shown in Table 7.1. In normal circumstances nisin does not significantly inhibit yeasts, molds, or Gram-negative bacteria. Its activity range encompasses, most importantly for its use as a food preservative, the Gram-positive endospore-forming genera that include *Bacillus* and *Clostridium*. It is active against both bacterial cells and their heat-resistant spores, enabling it to be used as a preservative in pasteurized or heat-treated foods that are not fully sterilized. Such heat treatments usually eliminate all vegetative cells so that such foods are vulnerable to spoilage only by these surviving spore-forming bacteria. Hurst (1981) reported that lower nisin levels prevent spore outgrowth compared with the levels required for inhibition of vegetative cells. Important pathogens in this group include *Bacillus cereus* and *Clostridium botulinum*. A relatively new species, *Bacillus sporothermodurans*, was first described in 1985 and, as its name suggests, this bacterium produces extremely heat-resistant spores capable of surviving ultra-high temperature (UHT) treatments. This species is extremely nisin sensitive. The growth of 10^4 cfu/ml of four different strains of this species was controlled for 7 days at 37°C by $0.125\ \mu\text{g}$ nisin/ml (Thomas and Delves-Broughton, 2001). Another important group of nisin-sensitive bacteria is lactic acid bacteria, organisms that may cause spoilage of low pH food such as salad dressings, sauces, or alcoholic beverages (e.g., wine and beer) that are often not heat processed. Nisin is also effective against another food-associated pathogen, *Listeria monocytogenes*. The meat spoilage organism *Brochothrix thermosphacta*, first described in 1951, is extremely nisin sensitive. After 7 days at 25°C , nisin at 0.625 to $2.5\ \mu\text{g}/\text{ml}$ prevented the growth of 10^3 cells/ml (Thomas and Delves-Broughton, 2001).

Wide variation in nisin sensitivities has been reported, with considerable variation being observed even between strains of the same species (Gupta and Prasad, 1989a). For example, Figure 7.2 shows the activity of nisin against three different strains of *Bacillus*. The outcome of nisin activity can also vary from achieving total kill of the cells to a growth inhibitory effect (Delves-Broughton et al., 1996). Nisin has a stronger bactericidal effect against energized cells, whereas

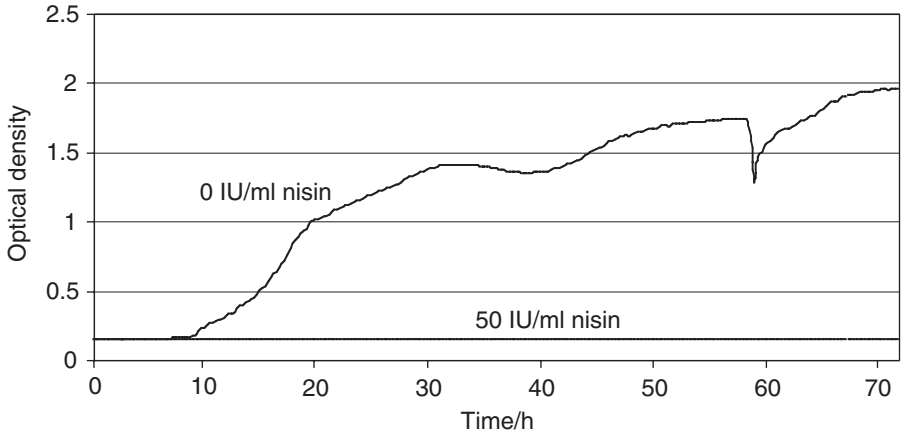
TABLE 7.1
Gram-Positive Species Sensitive to Nisin

Genus	Species	Description
<i>Alicyclobacillus</i>	<i>acidoterrestris</i>	Heat-resistant spore former. Growth at pH 2.5–6, 25°C–60°C. Spoilage organism of fresh/pasteurized fruit juice stored at ambient temperature.
<i>Bacillus</i>	<i>brevis, cereus, coagulans, licheniformis, macerans, megaterium, pumilis, subtilis, sporothermodurans, stearothermophilus</i>	Heat-resistant aerobic and facultative anaerobic spore formers. Includes psychrotrophs, thermophiles, acid-resistant, UHT-resistant, spoilage organisms (cause of “rope” in bread and flat sour spoilage), and food-poisoning pathogens.
<i>Brochothrix</i>	<i>thermosphacta</i>	Heat-sensitive spoilage organisms of meat. Growth between 0°C–30°C. Very nisin sensitive.
<i>Clostridium</i>	<i>bifermantans, botulinum, butyricum, histolyticum, pasteurianum, perfringens, putrificum, sordelli, sporogenes, tertium, thermosaccharolyticum, tyrobutyricum</i>	Heat-resistant spore-forming obligate anaerobes. Cause spoilage (blowing, etc.) and food-poisoning.
<i>Desulfotomaculum</i>	<i>nigrificans</i>	Heat-resistant spore-forming thermophiles. Obligate anaerobes. Cause blackening of canned food.
<i>Enterococcus</i>	<i>faecalis, faecium</i>	Aerobes/anaerobes. Varied nisin sensitivity. Cause spoilage.
<i>Lactobacillus</i>	Spp., <i>bulgaricus, brevis, buchneri, casei, curvatus, helveticus, fermentum, lactis, plantarum</i>	Cause spoilage of acid products, salad dressings, wine, beer, cider, cured meat products. Can grow at low pH. Some used for cheese production.
<i>Leuconostoc</i>	<i>oenos, mesenteroides</i>	Aerobes/anaerobes
<i>Listeria</i>	<i>innocua, monocytogenes</i>	Aerobes/anaerobes. Varied nisin sensitivity. Cause spoilage of wine and vegetables, producing slime.
<i>Micrococcus</i>	<i>luteus, varians</i>	Aerobes. <i>M. luteus</i> is indicator strain for the bioassay.
<i>Pediococcus</i>	Spp. <i>damnosus, pentosaceus</i>	Aerobes/anaerobes. Cause spoilage of wine and beer.
<i>Sporolactobacillus</i>	<i>inulinus</i>	Aerobe/anaerobe. Spore forming. Growth at low pH.
<i>Staphylococcus</i>	<i>aureus</i>	Aerobe/anaerobe. Varied sensitivity. Causes food poisoning.

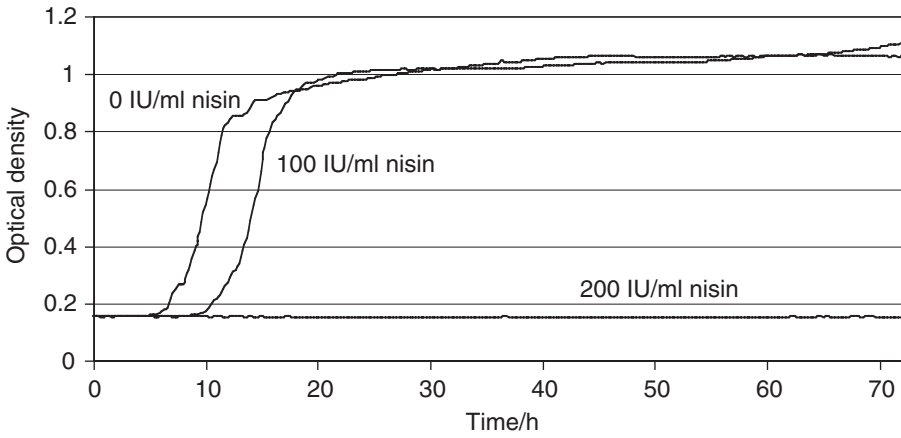
Source: From Thomas et al., 2000.

quiescent cells may survive but their growth will be inhibited (Sahl, 1991; Maisnier-Patin et al., 1992). As a result of the application of the hurdle theory of food preservation (Leistner and Gorris, 1995), cells will usually be nonenergized in foods because they may well be experiencing adverse conditions caused by low temperature, low pH, low water activity, poor nutrient availability, and the like. Nisin activity against spores is predominantly bacteriostatic.

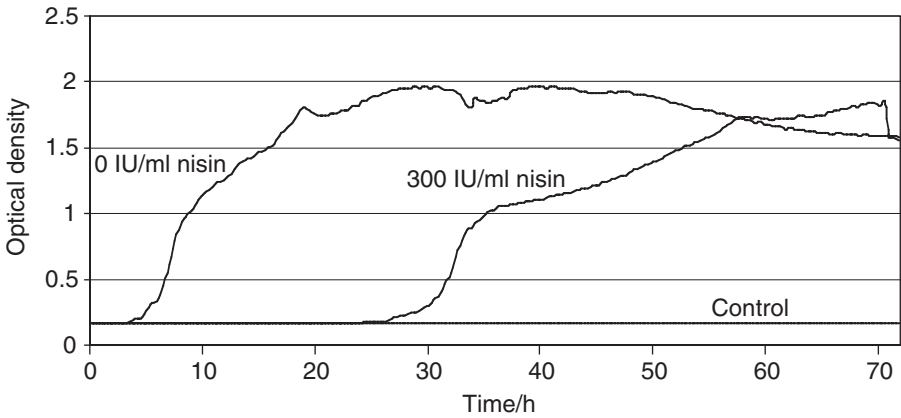
Gram-negative bacteria are resistant to nisin because the antimicrobial is unable to penetrate their complex cell wall to reach its site of action: the cytoplasmic membrane. There have been



(A)



(B)



(C)

FIGURE 7.2 Growth curve data obtained using an automated Microbiology Reader, the Bioscreen C. Strains were inoculated as 10^3 spores/ml and grown for 72 hours at 30°C in brain heart infusion medium containing different nisin levels. A: *B. subtilis* strains CMPD; B: *B. cereus* strain 204; C: *B. cereus* strain 3.046.

reports of nisin activity against *Neisseria* and *Arcobacter* (Mattick and Hirsch, 1947; Phillips, 1999). Nisin is also unable to penetrate yeast cell walls; therefore nisin can be used in food or beverages prepared by yeast fermentation. Nisin works in a concentration-dependent manner; consequently, heavily contaminated food will require higher nisin levels for adequate preservation (Rayman et al., 1981; Scott and Taylor, 1981b). Thus it is difficult to disguise poor-quality ingredients with nisin use. For example, canned peas containing 810 *Bacillus stearothermophilus* spores required twice as much nisin as similar containers containing 141 spores (Porretta et al., 1966).

MODE OF ACTION

Vegetative Cells

Nisin acts on energized vegetative cells (and membrane vesicles) by inserting into the membrane, forming pores, and dissipating the proton motive force. This inhibits uptake of amino acids and promotes rapid efflux of small metabolites, ions, or cytoplasmic solutes such as amino acids and nucleotides (Jung and Sahl, 1991; Abee, 1995). Abee et al. (1994) demonstrated that the cytoplasmic membrane is the primary target for nisin in experiments in which *L. monocytogenes* was treated with nisin Z. This resulted in immediate loss of cellular potassium ions, depolarization of the cytoplasmic membrane, and the hydrolysis and efflux of cellular ATP. The interaction of nisin with membranes and its pore-forming ability have been studied extensively. Nisin may also inhibit cell-wall synthesis, affect the activity of autolytic enzymes, and inhibit the outgrowth of bacterial spores (Bierman and Sahl, 1991; Hurst, 1981; Morris et al., 1984; Jung and Sahl, 1991; Ray, 1992).

Nisin preferentially interacts with negatively charged membranes (Garcia-Garcera et al., 1993; Driessen et al., 1995; Demel et al., 1996). Mutagenesis of nisin's charged amino acids demonstrated the importance of the positive charges in the carboxyl terminal region for membrane binding. One study systematically varied the anionic lipid content of the membranes and found that the amount of nisin binding increased dramatically at levels above 40% anionic lipid content (Demel et al., 1996; Breukink et al., 1997; van Kraaij et al., 1997). This indicated that nisin needed a relatively large amount of anionic lipids for efficient binding (Breukink and de Kruijff, 1999). Studies have also shown that increased nisin activity occurs in acidic conditions. Both nisin A and nisin Z have been shown to be capable of permeabilizing membranes whose membrane potential was absent or very low (Gao et al., 1991; Garcia-Garcera et al., 1993; Abee et al., 1994).

Driessen et al. (1995) described a "wedge model" that proposed that after binding, nisin inserts into the membrane without losing contact with the surface, forming transient pores. The phospholipid composition of the membrane determines this mode of action. It was suggested that nisin acted, even in the absence of a proton motive force, as an anion-selective carrier in liposomes composed of phosphatidylcholine. Nisin was believed to act against phospholipids such as phosphatidylglycerol, in a manner involving the local perturbation of the bilayer membrane structure and insertion of nisin in the membrane that was dependent on the transmembrane potential (inside-negative). It was also postulated that electrostatic interactions between nisin molecules and the phospholipids could bring the lipid head groups into the pore lining. This model worked well for model membrane experiments but did not explain how much lower levels of nisin cause inhibition *in vivo*. A very significant discovery was that nisin interacts with the peptidoglycan precursor, lipid II (Breukink et al., 1999; Wiedemann et al., 2001), which acts as a docking molecule for subsequent pore formation. Nisin forms ion-conducting pores in black lipid membranes. The presence of lipid II in such membranes enhances the ability of nisin to depolarize the transmembrane potential. In the presence of lipid II the transient lifetime of the pores increased from milliseconds to approximately 6 seconds, and the pore size increased from approximately 1 nm to 2.0 to 2.5 nm (Wiedeman et al., 1998). Cells containing low levels of lipid II were shown to be less sensitive to nisin (Brotz et al., 1998). Nisin mutant peptides have since been identified that were unable to form pores but retained considerable antimicrobial activity by blocking the incorporation of lipid II into

peptidoglycan, thus inhibiting cell-wall synthesis (Wiedemann et al., 2001). This demonstrated that nisin has two potentially cidal mechanisms.

Budde and Jakobsen (2000) examined real-time interactions between nisin and cells on a solid surface. The method was based on fluorescence ratio-imaging microscopy and measurements of changes in the intracellular pH of carboxyfluorescein succinimidyl ester-stained cells during exposure to nisin. In the absence of nisin the intracellular pH of the cells was almost constant at pH 8.0, and independent of the external pH that ranged from pH 5 to 9. In the presence of nisin, dissipation of the pH gradient was observed, which was a function of nisin and time. In this system, immobilized cells were significantly less sensitive to nisin than planktonic cells.

The outer membrane of Gram-negative bacteria effectively excludes nisin from making contact and interacting with the cytoplasmic membrane (Kordel et al., 1989). In combination with a chelating agent, such as disodium ethylenediamine tetraacetic acid (EDTA), nisin can be effective against Gram-negative bacteria such as *Salmonella*, *Escherichia coli*, and other members of the Enterobacteriaceae family (Kordel and Sahl, 1986; Blackburn et al., 1989; Stevens et al., 1991, 1992; Vaara, 1992; Delves-Broughton, 1993; Cutter and Siragusa, 1995a). The chelating agents remove divalent cations from the Gram-negative walls, releasing phospholipids and lipoproteins and increasing cell-wall permeability. Boziaris and Adams (1999) used a model substrate to examine combinations of chelators and nisin generated *in situ* to inactivate *E. coli* and other Gram-negative bacteria in a model substrate. Only EDTA and pyrophosphate were able to cause appreciable inhibition when nisin was added exogenously. There was no inhibition observed when nisin was produced *in situ*; this was attributed to the poor chelating power of EDTA under the acid conditions caused by the *Lactococcus* growth. Unfortunately chelating agents are much less effective in food as a result of their preferential binding to free divalent ions within the food. Any treatment, such as sublethal heat, hydrostatic pressure, or freezing, or organic acids, which disrupts the outer membrane, may render Gram-negative cells sensitive to nisin (Blackburn et al., 1989; Kalchayanand et al., 1992; Kordel and Sahl, 1986; Hauben et al., 1996). Rapid chilling in the presence of nisin caused a dose-dependent reduction in the populations of several Gram-negative bacteria, despite the fact that appreciable injury to the outer membrane was not detected (Boziaris and Adams, 2000). This was attributed to a transient susceptibility caused by phase changes in the lipids associated with the outer membrane, which were rapidly reversed when the cells returned to higher temperature. Removal of the cell wall of yeasts has been shown to facilitate nisin access to the cytoplasmic membrane causing cell rupture (Dielbandhosing et al., 1998). Cells were most sensitive to nisin in the S phase, when the permeability to fluorescein isothiocyanate dextrans was shown to be at its highest.

It has been observed that exposure to nisin can result in the adaptation of bacterial cells to high nisin concentrations. Ming and Daeschel (1993) grew *L. monocytogenes* cells in laboratory medium containing nisin at concentrations 2 to 8 times the minimum inhibitory concentration (MIC) values and found spontaneous resistance occurring at a frequency of 10^{-6} to 10^{-8} . Examination of one resistant strain revealed that changes had occurred in the bacterial membrane. The mutant had a higher phase transition temperature, more straight-chain fatty acids, and less branched-chain fatty acids resulting in a decreased efficiency of nisin pore formation, a phenomenon also observed by Davies and Adams (1994). Nisin-resistant variants have also shown increased cell wall hydrophobicity and visible thickening of the cell wall (Maisnier-Patin and Richard, 1996). Davies et al. (1996) also reported a correlation between cell surface hydrophobicity of *L. monocytogenes* replace with nisin sensitivity. Studies investigating the effects of environmental conditions (pH, salt, and temperature) have generated results indicating that the occurrence of nisin-resistant *L. monocytogenes* in foods would not be a problem (de Martinis et al., 1997). Gravesen and Knochel (1988) examined *L. monocytogenes* isolates from various sources and found natural resistance occurred rarely.

Spores

In general (endo)spore-forming bacteria such as *Bacillus* and *Clostridium* are very sensitive to nisin. Spores are usually more sensitive than cells, and sensitivity is increased by acidic conditions and spore-damaging heat treatments. For example, as early as 1956 O'Brien et al. reported that *Bacillus coagulans* and *B. stearothermophilus* were very nisin sensitive and confirmed earlier reports that low nisin concentrations appeared to increase the heat sensitivity of spores. At the time, this was attributed to the carryover of nisin into the plating medium. Spores of *Clostridium* PA 3679 that survived a heat treatment of 3 minutes at 121°C ($F_0 = 3$) were ten times more sensitive to nisin compare to those that were not heat treated. It has also been shown that spores and cells of thermophilic spore formers such as *B. stearothermophilus* and *Clostridium thermosaccharolyticum* are extremely heat sensitive compared to mesophilic spore formers (Jarvis, 1967).

The outcome of nisin activity on spores is usually sporostatic, although sporocidal effects have been reported depending on the target strain and extent of heat treatment (de Vuyst and Vandamme, 1994). Nisin does not prevent germination but prevents postgermination swelling and subsequent outgrowth of the spore (Hitchins et al., 1963; Gould, 1964). Gould and Hurst (1962) classified *Bacillus* spores into two groups. The small-spored species (e.g., *Bacillus subtilis*), which appeared to rupture their envelopes by mechanical pressure, were inhibited by about 0.125 µg/ml. The large-spored species (e.g., *B. cereus*) outgrew by a lytic mechanism. The spore envelope disappeared during outgrowth, and these spores were much more nisin resistant, requiring more than 2.5 µg/ml for inhibition. Jarvis (1967) showed that the spore envelope lytic enzyme was not a nisinase, and there was no quantitative relation between the production of the anti-nisin activity and the resistance of vegetative cells. Chan et al. (1996) produced a nisin mutant that had replacement of the dehydroalanine residue at position 5 by an alanine residue. This caused a reduction of nisin antisporic activity but did not affect its activity against vegetative cell, an effect that had also been observed with subtilin by Liu and Hansen (1992; 1993). The double bond of the Dha5 residue may be the point of interaction between nisin and a spore-associated factor essential for growth.

FACTORS AFFECTING ANTIMICROBIAL ACTIVITY

The outcome of nisin activity within a food system depends on numerous factors. Other preservative hurdles such as heat treatments, low water activity, modified atmosphere, low temperature, and pH enhance activity (Rogers and Montville, 1994; Thomas and Wimpenny, 1996; Beuchat et al., 1997; Blom et al., 1997; Datta and Benjamin, 1997; Szabo and Cahill, 1998; Ueckert et al., 1998). Cerrutti et al. (2001) used Doehlert design and surface methodology to analyze the effects of bacterial stress factors on nisin performance in food. Results indicated that there was a synergistic inhibitory effect between water activity and nisin. A reduction in *E. coli* numbers by up to 4 to 5 logs was achieved by up to 35 µg/g nisin, pH 5.5 to 6.5, and water activity between 0.97 and 0.98.

Nisin works better in liquid or homogeneous foods compared to solid or heterogeneous products because the bacteriocin can be better and more evenly distributed throughout the food matrix of the former. In our experience better nisin activity is observed in liquid laboratory media compared with actual food products; however, food ingredients can sometimes afford protection to nisin during heat processing. Because of nisin's hydrophobic nature, fat in food may hinder its uniform distribution and render it unavailable for activity (Jung et al., 1992). Certain food additives should be avoided in foods preserved with nisin. For example, nisin is degraded in the presence of sodium metabisulphite (used as an antioxidant, bleach, and antimicrobial) and titanium dioxide (used as a whitener). Polyvalent cations such as Ca^{2+} , Mg^{2+} , or Gd^{3+} have been shown to reduce the efficiency of nisin Z against *L. monocytogenes* (Abee et al., 1994). It is thought that these ions may interact with the negatively charged phospholipid headgroups of phosphatidylglycerol and cardiolipin in the cytoplasmic membrane (Harwood and Russell, 1984; O'Leary and Wilkinson, 1988).

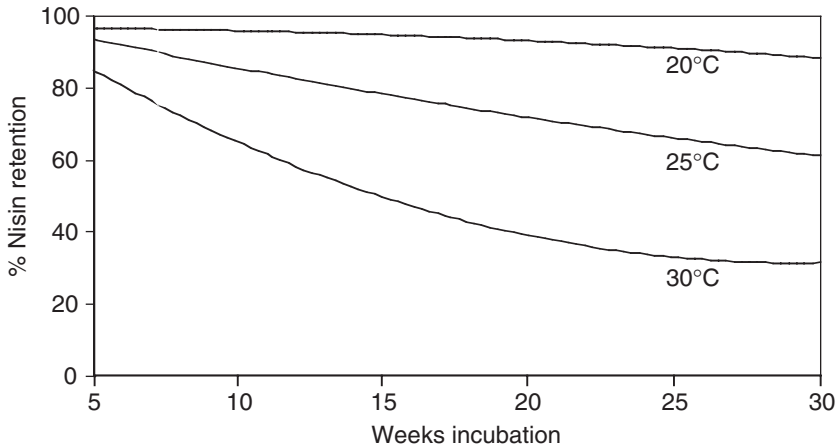


FIGURE 7.3 Retention of nisin in processed cheese stored at different temperatures.

In food, a certain proportion of nisin will be lost after heat treatment and with time during storage. During the melting process used in processed cheese manufacture, as much as 20% nisin will be lost and nisin additional levels need to be adjusted to compensate for this. The degree of nisin loss depends on the extent of heat treatment, the period of time the cheese is kept molten, and the pH of the product. Nisin loss is considerable if a UHT process is involved — up to 40% may be lost. However, much less nisin will subsequently be needed to control the reduced number of heat-damaged spores that can survive this treatment.

Loss during storage was first observed by Hirsch (1951) in Swiss-type cheese made with a nisin-producing starter culture. Decline of nisin levels during storage is temperature dependent (Figure 7.3). Levels remain relatively stable during refrigerated storage, but loss is faster at ambient temperatures (Delves-Broughton, 1990). Higher nisin levels (12.5 to 15.5 $\mu\text{g/g}$) are needed for products stored at high ambient temperatures for long periods. However, nisin does offer the possibility of avoiding storing products including processed cheese at chill temperature. This can be important when cold storage distribution chains cannot be guaranteed or when refrigerated storage is not possible.

Temperature may also affect the activity of nisin. Abee et al. (1994) found that the rate of nisin Z-induced K^+ efflux from listerial cells grown at 30°C was reduced when the temperature was lowered. This was attributed to a decrease in membrane fluidity caused by the temperature reduction. *Listeria* cells can adapt to growth at low temperatures by increasing the fluidity of the membrane by increasing the proportion of short and/or branched fatty acyl chains of the lipids (Russell et al., 1995). Nisin Z was observed for cells adapted and grown at 4°C (Abee et al. 1994). This concurs with reported nisin Z efficacy against foodborne bacteria grown in laboratory medium, in low-fat milk, and at high and low temperatures (Abee et al., 1995). Many foods are stored at low temperatures. It seems from this evidence that if bacteria are capable of growth at these low temperatures, their membranes will have adapted and will be sensitive to nisin action.

In foods that are not heat treated, or that have been minimally processed, nisin may be degraded during storage by proteolytic enzymes derived from microbial, plant, or animal cells within the food. Enzymes capable of degrading nisin include pancreatin, α -chymotrypsin, and subtilopeptidase inactivate (Heinemann and Williams, 1966; Jarvis, 1967; Jarvis and Mahoney, 1969). Nisinase, a specific enzyme that target nisin, has been reported from a few strains exhibiting reduced nisin sensitivity. The enzyme was isolated from *Bacillus* and proved to be dehydropeptide reductase (Kooy, 1952; Carlson and Bauer, 1957; Lipinska and Strzalkowska, 1959; Alifax and Chevalier,

1962; Jarvis, 1966; 1967; Jarvis and Farr, 1971; Lipinska, 1977; Hurst, 1981; Collins-Thompson et al., 1985; Ray, 1992).

The effectiveness of nisin decreases with the size of the inoculum (Scott and Taylor, 1981b; Rayman et al., 1981). Ramseier (1960) published a figure illustrating this point with spores of *Clostridium butyricum*. He found a close relation between the nisin concentrations required to inhibit outgrowth and spore load. Increasing the spore load 10-fold requires an increased nisin concentration of about half a logarithmic cycle expressed in international units (Ramseier, 1960). Prevention of the outgrowth of *C. botulinum* requires 10 to 100 times more nisin than that needed to prevent the outgrowth of *C. butyricum*.

The solubility and stability of nisin solutions decrease as the pH increases. Ramseier (1960) reported that the effectiveness of nisin increased with increasing pH, but the weight of evidence points in the opposite direction. Rayman et al. (1981) showed that the effectiveness of nisin decreased with increasing pH, and Scott and Taylor (1981a,b) reported similar results.

Certain compounds are synergistic with nisin. These include essential oils (carvacrol, thymol, and carvone), organic acids, bacteriocins, garlic extract, lysozyme, chelating agents, lactoperoxidase, nonionic, and amphoteric surfactants and emulsifiers such as monoglycerides (particularly monolaurin) and sucrose fatty acid esters (Blackburn et al., 1989; Monticello, 1989; Oscroft et al., 1990; Proctor and Cunningham, 1993; Woolsey et al., 1994; Buncic et al., 1995; Avery and Buncic, 1997; Miles et al., 1997; Thomas et al., 1998; Zapico et al., 1998; Mansour et al., 1999; Pol and Smid, 1999; Boussouel et al., 2000; Chung and Hancock, 2000; Thomas et al., 2000; Mansour and Milliere, 2001; Pol, 2001; Singh et al., 2001). Protective cultures producing other bacteriocins *in situ* may also enhance nisin activity (Hanlin et al., 1993; Schillinger, 1999). Future new preservative systems are likely to use combinations of agents to enhance the preservative effect and increase the antimicrobial spectrum of nisin. This type of combined approach has already proved successful. For example, a combination of nisin, lysozyme, and citrate was effective against *L. monocytogenes* in liver pâté and against this organism and spoilage *Bacillus* in processed cheese (ter Steeg, 1993; ter Steeg et al., 1994).

Novel nonthermal pasteurization methods such as ultrahigh pressure and electroporation (pulsed electric field) are also being investigated in combination with nisin. For example, pulsed electric field has been shown to enhance the cidal action of nisin against *B. cereus* and *M. luteus* (Dutreux et al., 2000; Pol et al. 2000). In contrast, an *E. coli* study by Terebiznik et al. (2000) reported nisin inactivation by pulsed electric fields and that nisin was protected by the presence of bacterial cells. Kalchayanand et al. (1994) found that ultra high pressure and electroporation injured cells of *L. monocytogenes*, *E. coli*, and *Salmonella* Typhimurium such that they became more susceptible to nisin. Hauben et al. (1996) showed that ultrahigh pressure sensitized *E. coli* to nisin and postulated that this was the result of disruption of the outer membrane. Masschalck et al. (2000, 2001) later confirmed this effect. A synergy between nisin and ultrahigh pressure against *Bacillus* and *Clostridium* spores has been reported (Roberts and Hoover, 1996; Stewart et al., 2000). A combination of nisin and sublethal ultra high pressure was synergistic against a yeast strain (Ter Steeg et al., 1999).

APPLICATIONS

The applications of nisin in food preservation have been extensively covered in several reviews (Hurst, 1981; Delves-Broughton, 1990; de Vuyst and Vandamme, 1994; Delves-Broughton and Gasson, 1994; Thomas et al., 2000; Thomas and Delves-Broughton, 2001). Nisin can be used in a wide range of liquid or solid foods, cans or packages, stored at ambient or chill temperature. Its usage is mainly in heat-processed foods to prevent spoilage by spore-forming bacteria, but it is also used to control spoilage by heat-sensitive lactic acid bacteria and *B. thermosphacta*. It is also used as part of the safety measures to control pathogens such as *B. cereus*, *L. monocytogenes*, and *C. botulinum*. There are several good reasons to use nisin as a preservative in food. For example, it can extend the shelf life of both chilled and ambient stored food. It can protect chilled foods

TABLE 7.2
Typical Addition Levels of Nisin and the Commercial Extract Nisaplin

Food	Nisin (vg/g)	Nisaplin (mg/kg, mg/L)	Typical Target Organism
Processed cheese	2.5–15	100–600	<i>Bacillus</i> , <i>Clostridium</i>
Milk and milk products	0.25–1.25	10–50	<i>Bacillus</i> (<i>B. sporothermodurans</i>)
Pasteurized chilled dairy desserts	1.88–5.0	75–200	<i>Bacillus</i> , <i>Clostridium</i>
Liquid egg	1.25–5	5–200	<i>Bacillus</i> (<i>B. cereus</i>)
Pasteurized soups	2.5–6.25	100–250	<i>Bacillus</i>
Crumpets	3.75–6.25	150–250	<i>B. cereus</i>
Fruit juice (pasteurized/ambient storage)	0.75–1.5	30–60	<i>Alicyclobacillus acidoterrestris</i>
Canned food	2.5–5	100–200	<i>B. stearothermophilus</i> , <i>Cl. thermosaccharolyticum</i> , <i>Cl. botulinum</i>
Dressings and sauces	1.25–5	50–200	Lactic acid bacteria, <i>Clostridium</i> , <i>Bacillus</i>
Meat products such as bologna, frankfurter sausages	5–10	200–400	Lactic acid bacteria <i>Br. thermosphacta</i> <i>L. monocytogenes</i>
Ricotta cheese	2.5–5	100–200	<i>L. monocytogenes</i>
Beer, wine, fermented beverages, spirits			Lactic acid bacteria (<i>Lactobacillus</i> , <i>Pediococcus</i>)
Pitching yeast wash	25–37.5	1000–1500	
Reduced pasteurization	0.25–1.25	10–50	
During fermentation	0.63–2.5	25–100	
Postfermentation	0.25–1.25	10–50	

Source: Updated from Thomas et al., 2000.

from temperature abuse. It can enable thermal processing treatments to be reduced (if this is legally permissible). This has the consequence of reducing the negative effects heat treatments have on product quality and reducing manufacturing costs. Distribution costs can also be reduced because nisin may eliminate the need for chilled transport.

Additional levels depend on the type of food, heat processing, pH, storage conditions, bacterial load, and the required shelf life. The commercial extract Nisaplin® is usually added at 10 to 750 mg/kg, equivalent to nisin at 0.25 to 18.7 µg/g. Typical addition levels are shown in Table 7.2. Nisin is effective in a wide range of pH values (pH 3.5 to 8.0). Nisin is very effective in pasteurized liquid egg, which typically has a pH of 7.3 to 7.8. This refutes the assertion, often quoted, that nisin is not effective at neutral pH values.

DAIRY APPLICATIONS

Pasteurized Processed Cheese

Hirsch et al. (1951) were the first to use nisin as a food preservative when they made Swiss-type cheese with nisin-producing starter culture, which effectively prevented “blowing” caused by *C. butyricum* and *Clostridium tyrobutyricum*. Subsequently they used a similar technique for the preservation of processed cheese (McClintock et al., 1952). The promising results stimulated nisin manufacture and the subsequent development of a food preservative-grade preparation. The legal precedent for use of nisin in U.S. foods was set with pasteurized processed cheese spreads (FDA, 1988). The greatest use of nisin still remains in pasteurized processed cheese and associated products

such as spreads and slices. The anaerobic environment, high moisture (ranging from a minimum of 44% in block cheese to 60% to 65% in spreads, sauces, or dips), and pH range 5.4 to 6.0 usually found in processed cheese favor the growth of *Clostridium* species, in particular *C. butyricum*, *C. tyrobutyricum*, and *Clostridium sporogenes* (Meyer, 1973; Thomas, 1977). Facultatively anaerobic *Bacillus* may also cause problems. Spoilage is observed as blowing, off-odors, and liquefaction. Incidence of *C. botulinum* is rare but is possible, particularly when temperature abuse occurs (Kosikowski, 1977; Briozzo et al., 1983; Collins-Thompson and Woods, 1992). Somers and Taylor (1987) found water activity and the levels of sodium chloride and phosphate to substantially affect the minimum concentration of nisin for inhibition of *C. botulinum* types A and B. Tanaka et al. (1986) found the pH of the pasteurized cheese spread; the level of both sodium chloride and phosphate affected the required amount of nisin needed for the antibotulinal effect. Higher nisin levels are required in formulations with high moisture content and low levels of sodium chloride and phosphate. The bioload of contaminant spores and the period and temperature of shelf life also need to be considered when deciding nisin addition levels.

The ingredients of processed cheese and similar products include cheese; butter; whey powder; skimmed milk powder; emulsifying salts (phosphate or citrate); and possibly flavor ingredients such as spices, herbs, onions, mushrooms, shellfish, or meat (Meyer, 1973; Thomas, 1977; Zehren and Nusbaum, 1992; Delves-Broughton, 1998). Some of these ingredients, in particular those of dairy origin and herbs and spices, may introduce significant levels of bacterial spores that can survive the heat process used to melt the cheese. This is typically 85°C to 105°C for 5 to 10 minutes. The practice of feeding silage to the dairy cattle has been linked to high spore contamination (Glaeser 1989).

Nisin, at concentrations of 2.5 to 6.24 µg/g, was shown to be extremely effective in preventing growth of relevant *Clostridium* species inoculated in processed cheese and cheese spreads (with or without ham) incubated at 37°C (Delves-Broughton and Gasson, 1994). This effectiveness was also reported by Plocková et al. (1996) who found similar nisin levels inhibited *Clostridium* and *Bacillus* in processed cheese during a 3-month storage period. These are just two examples of many such trials. Nisin at 4 to 5.1 µg/g also reduced spoilage microflora in processed cheese analogs incorporating fat substitutes over a 4-month storage at 5°C (Muir et al., 1999).

In the manufacture of processed cheese nisin is usually added to the heated cheese at the same time as the melting salts (Fowler and McCann, 1971). Nisin should not be mixed with acid correctant emulsifying salts, which are typically at pH values >9. Nisin efficacy depends on the number and type of clostridial spores present in the product (Fowler, 1971). For low spore numbers (<10 per g) the minimum effective nisin level was considered to be 2.5 to 6.25 µg/g and 1.75 µg/g for butyric clostridia. An approximate rule for processed cheese is that twice as much nisin is required for every 10-fold increase in spore numbers. Effective levels are also related to the actual storage temperature of the product and the required shelf life. Sufficient nisin must remain in the product during this shelf life to prevent growth of the spores because nisin activity is sporostatic but not sporocidal. Typical losses during the melt process are 15% to 20%, but this is dependent on pH, the extent of heat process, and the time the cheese is held at molten temperatures before packing and cooling (Delves-Broughton, 1990). Nisin efficacy may be reduced if the processed cheese is not allowed sufficient cooling postprocessing.

Natural Cheese

It was rapidly realized after the early work of Hirsch et al. (1951) that a major problem with nisin use in cheese was that its inhibition of the bacterial starter cultures used for ripening and production of cheese characteristics (Kooy, 1952; Kooy and Pette, 1952; Lipinska, 1956). To overcome this, starter cultures had to be trained to grow in the presence of nisin; however, although this was achieved, it proved impractical (Lipinska, 1977). Recent developments in gene technology have revived interest in this application (Eckner, 1991; Hugenholtz and de Veer, 1991; Roberts et al., 1992; Maisnier-Patin et al., 1992; Yezzi et al., 1993; Delves-Broughton et al., 1996; Rodriguez

et al., 2001). Nisin is encoded as a transposon and therefore can be moved from cell to cell relatively easily (Rauch and de Vos, 1992). A nisin-producing starter culture for Gouda cheese was developed by a Dutch group (Hugenholtz and de Veer, 1991; Hugenholtz et al., 1995). An improvement from earlier attempts was to use lactose-negative strains as donor cells, enabling the selection of transconjugants for analysis of good cheese sensory characteristics. Cheese was prepared with different nisin levels by using nisin-producing and nisin-immune strains in different ratios as starters. The nisin produced within the cheese protected against growth of *C. tyrobutyricum* and *Staphylococcus aureus* during ripening. A nisin-producing transconjugant *L. lactis* subspecies *cremoris* has also been used as a starter culture in a pH-controlled bulk Cheddar cheese starter culture system. A high level of 17.5 µg/g nisin was produced (Roberts et al., 1992; Yezzi et al., 1993). Such nisin-containing cheese has been used to make processed cheese spreads, which showed better protection against *C. sporogenes* in inoculated trials (Roberts and Zottola, 1993).

Nisin has been shown to be effective against *L. monocytogenes* in surface mold-ripened soft cheeses. Nisin (at 0.625 to 2.5 µg/ml) was added to the milk, which was then given a mild heat treatment before addition of nisin-producing starter cultures. In challenge studies, a 5- to 6-log reduction of the pathogen was achieved (Maisier-Patin et al., 1992; Richard, 1993).

Soft white cheese such as ricotta, paneer, queso fresco, and fresh Hispanic-style cheeses are not made using starter cultures, and nisin can therefore be used in these cheeses without causing problems with their production. Davies et al. (1997) investigated the efficacy of nisin added directly to the milk used to prepare ricotta cheese by direct acidification. The growth of inoculated *L. monocytogenes* was inhibited during storage at 6°C to 8°C for longer than 8 weeks. Control cheese not containing nisin contained unsafe levels of the pathogen after 2 weeks. Nisin bound preferentially to the curd with minimum losses in the whey. Similar success has been reported in queso fresco cheese, with nisin produced *in situ* in a pre-fermentation step with a *L. lactis* subspecies *lactis* producer strains or by salting nisin onto the finished curd (Degnan et al., 1996). Nisin, added at a level of 50 µg/g to cottage cheese after production, caused a 3-log reduction of inoculated *L. monocytogenes* in 3 days at 20°C. Controls showed only a 1-log reduction after 7 days (Ferreira and Lund, 1996). Ajao et al. (1992) have also reported good control of *L. monocytogenes* by nisin in cold-pack blended cheese (i.e., not heat processed).

Pasteurized Milk and Other Dairy Products

Interest in nisin use in these products often comes from countries with warm climates that may have inadequate refrigeration facilities and refrigerated transport. Nisin can protect against temperature abuse and extend shelf life. Milk pasteurized by either 72°C for 15 seconds or 115°C for 2 seconds reportedly had greatly increased shelf life at 10°C because of addition of nisin at 1 µg/ml (Wirjantoro and Lewis, 1996). Earlier studies (Fowler and Gasson, 1991) had reported extension of shelf life of pasteurized whole milk by more than 6 days at 15°C and 2 days at 20°C as a result of nisin addition at 0.5 to 10 µg/ml. Another benefit is improvement of product quality by nisin addition enabling a reduction of heat treatments. A further study by Wirjantoro et al. (2001) demonstrated the effectiveness of a reduced heat treatment (117°C for 2 seconds) and nisin (1.88 and 3.75 µg/ml). The milk had an extended shelf life at refrigeration temperature, and its quality was considerably better than milk subjected to UHT processing of 142°C for 2 seconds. Thermophilic heat-resistant spores may survive in “commercially sterile” milk that has been retorted or autoclaved; these spores are nisin-sensitive. A study of milk processed at 121°C for 11 to 13 minutes found that, after 10 days at 55°C, 30% to 100% of milk had spoiled unless nisin had been added at 0.75 to 1.25 µg/ml (Pesquera, 1966). Similar efficacy was reported by Wajid and Kalra (1976), describing increase of shelf life of “sterilized” cows’ milk to 60 days compared to 3 to 7 days without nisin. Shehata et al. (1976) successfully used low levels of nisin as an adjunct in the production of sterile whole buffalo milk or chocolate milk. They were able to reduce the heat processing by about 80% and reported satisfactory storage for 21 days at 37°C.

Flavored milk such as chocolate milk can have higher numbers of contaminating spores derived from the flavoring ingredients. Nisin at 2.5 $\mu\text{g}/\text{ml}$ was effective in controlling spoilage of such milk inoculated with spores of the mesophile *Clostridium* PA3679 and thermophile *B. stearothermophilus*, prior to a heat treatment at an F_0 value of 3.2. Nisin at 1 $\mu\text{g}/\text{ml}$ also prevented spoilage of chocolate milk in a large-scale trial for 3 weeks at 55°C and 6 months at ambient temperature (Heinemann et al., 1965). Similar effectiveness has been reported by Tramer (1964), Shehata et al. (1976), and Pesquera (1966).

Nisin can also be used to preserve pasteurized dairy desserts that cannot be sterilized because of consequent heat damage (Anonymous, 1985). The shelf life of crème caramel puddings was extended from <6 days to >35 days at 12°C by addition of nisin at 1.25 and 3.75 $\mu\text{g}/\text{g}$. Similar efficacy was shown for chocolate dairy desserts. In dairy desserts and milks, nisin can be added to a small quantity of milk, mixed into the bulk milk, filled, and processed (Fowler and McCann, 1971). As with all nisin applications, efficacy is best in homogeneous products. Phillips et al. (1983) reported extension of the shelf life of pasteurized double cream with nisin at 0.625 to 2.5 $\mu\text{g}/\text{ml}$. The shelf life was compromised by postprocessing contamination with Gram-negative bacteria. In the same paper it was claimed that nisin increased the growth rate of Gram-negative bacteria. Recent investigations in Danisco laboratories refute this claim. The growth curves of 14 Gram-negative bacteria were analyzed in the presence of 2.5 to 12.5 $\mu\text{g}/\text{ml}$ nisin. None of the strains showed an increase in the growth rate in the presence of nisin; all the strains showed slight growth inhibition at the higher nisin concentrations (Ingram and Thomas, unpublished results). Nisin has also been used in kheer, an Indian dessert obtained from milk, sugar, and rice (Sukumar et al., 1976). This product had a shelf life of 2 to 3 days at 37°C; with 5 $\mu\text{g}/\text{g}$ nisin the shelf life increased to 8 to 10 days.

Nisin at 14 mg/g has been shown to reduce numbers of *Listeria* in ice cream during the manufacturing process and frozen storage at -18°C for 3 months (Dean and Zottola, 1996). This was observed in both 1% and 3% fat ice cream and is noteworthy because nisin activity occurred at such low temperature. Nissen et al. (2001) studied the germination and growth of both inoculated and naturally occurring *Bacillus* strains in heat-treated cream with and without nisin. In heat-treated cream (90°C for 15 minutes), bacterial growth was mainly the result of *B. pumilus*, *B. licheniformis*, and other *Bacillus* species. *B. cereus* was also isolated. Bacterial growth during storage of the cream at 8°C or 10°C was completely inhibited by low concentrations of nisin. It was concluded that the safety of cream, for instance in “ready-to-eat” products, could be improved by use of nisin.

Despite the problems associated with nisin use in yogurt (inhibition of starter bacteria and consequent delay of fermentation), several studies have reported good results. Use of nisin at 0.625 to 1.25 $\mu\text{g}/\text{g}$ was recommended by Bayoumi (1991). This produced acceptable yogurt after 5.5 hours that had good organoleptic and textural characteristics and a shelf life of 19 days at 10°C or 33 days at 6°C. Prevention of overacidity can be achieved by mixing nisin into stirred yogurt when the required fermentation is completed (Gupta and Prasad, 1988; 1989b,c); this has been confirmed in our own laboratories (Thomas et al., 2001; Delves-Broughton, 2001). Nisin has also been used in other fermented milk products, such as “nono,” a Nigerian drink. Extension of shelf life at 25°C and 8°C was achieved by addition of Nisaplin® after fermentation (Olasupo et al., 1996).

CANNED FOODS

Nisin efficacy in canned vegetables was first reported by Gillespy in 1953, who detailed successful nisin trials with canned beans in tomato sauce inoculated with *C. thermosaccharolyticum* and found no spoilage when nisin was used at a concentration of 5 $\mu\text{g}/\text{g}$.

Low-acid canned foods having a pH greater than 4.5 should normally receive a minimal heat treatment ($F_0 = 3$) to ensure the destruction of *C. botulinum*. Even at treatments higher than this, heat-resistant spores of the thermophilic bacteria *B. stearothermophilus* or *C. thermosaccharolyticum* may survive (Eyles and Richardson, 1988). Nisin can be used to control this spoilage, which

occurs particularly when cans are stored at high ambient temperatures for long periods. Nisin can also be used to decrease the heat treatment to reduce energy consumption and to improve the nutritional value, appearance, and texture of the food (Hurst, 1981).

C. botulinum cannot grow in high-acid food (<pH 4.5), and thus can be processed at F_0 levels below 3. Spoilage in these products can occur as a result of growth of acid-tolerant spore formers such as *C. pasteurianum* and *B. macerans*. The growth of such bacteria can be controlled by nisin and has been reported in high-acid foods such as canned tomatoes (Vas, 1963; Maslennikova et al., 1968) and tomato juice (Poretta et al., 1968).

For all these applications, nisin can be added as a liquid solution by combining this with a small volume of canning brine and then thoroughly mixing it into the bulk fluid. Nisin control of spoilage of canned vegetables has also been reported for asparagus (Hernandez et al., 1964), mushrooms (Funan et al., 1990), okra (El-Samehy and Elias, 1977), peas (Gillespy, 1953; Nekhotenova, 1961; Vas et al., 1967), peppers (Duran et al., 1964), and potatoes (Maslennikova and Loshina, 1968).

MEAT APPLICATIONS

There are many reported investigations of nisin use as a decontaminant in meat and meat products. Efficacy is variable and appears to depend on the nature of the product and the specific target bacterium.

Raw Meat

Several studies have focused on nisin use as a carcass disinfectant together with chelating agents, a combination known to act against Gram-negative bacteria and Gram-positive bacteria *in vitro* (Blackburn et al., 1989; Stevens et al., 1991, 1992; Delves-Broughton, 1993; Cutter and Siragusa, 1995a). The promising results observed in buffer systems did not translate successfully to raw meat treatment, either by spraying or dipping. An example of this was the work of Cutter and Siragusa (1995a, 1995b) who demonstrated good cidal effects against *E. coli* O157 and *S. Typhimurium* in a buffer system using a nisin/chelator combination. However, this treatment was much less effective when used as a treatment of lean beef tissue. In another study, nisin at 2.5 µg/ml achieved an effective kill of *L. monocytogenes* when used in scald water at 52°C for 3 minutes, but the effect was much less when the bacteria were located on the turkey skin (Mahadeo and Tatini, 1994). An optimum treatment of nisin (100 µg/ml) with a chelating agent (EDTA, citric acid) and a surfactant (Tween 20) was described by Shefet et al. (1995) for use on chicken drumsticks against *S. Typhimurium* and for extension of shelf life.

Cutter and Siragusa (1994) examined decontamination of raw meat surface using a pilot scale model carcass washer and achieved a reduction in total bacterial count and, in a further study, a reduction in *Listeria innocua*, *Carnobacterium divergens*, and *B. thermosphacta* was observed (Cutter and Siragusa, 1996b). This group also examined the effect of nisin incorporation into a surface coating of calcium alginate, which resulted in better reduction and control of *B. thermosphacta* on beef surfaces and better nisin retention (Cutter and Siragusa, 1996a). They then experimented with incorporating nisin into a commercially available binding system; this inhibited *B. thermosphacta* on a meat surface (Cutter and Siragusa, 1998). Successful inhibition of *B. thermosphacta* was again reported. Chung et al. (1989) investigated the effect of nisin added to raw meat inoculated with meat-associated bacteria. Nisin activity, when extracted from the meat, decreased by 95% during storage for 4 days at ambient temperature and by 70% at 5°C. The growth of the inoculated Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) was delayed but not that of the Gram-negative bacteria.

Nisin was included in a study to investigate control of *L. monocytogenes* in refrigerated and frozen ground beef (Park et al., 2001). Nisin achieved a greater reduction of bacterial numbers in

refrigerated beef compared to lacticin, with an even better reduction occurring in frozen beef. Yuste et al. (1998) reported that a combination of high hydrostatic pressure (350 Mpa), 100 µg/g nisin, and 1% glucono delta lactone extended the shelf life of mechanically recovered poultry meat during a 30-day chilled storage period.

Ariyapatitipun et al. (2000) demonstrated the multiple hurdle concept of food preservation by using a combination of lactic acid, nisin, polylactic acid, low temperatures, and vacuum packaging to inhibit growth of *L. monocytogenes* on raw beef for up to 42 days. Pawar et al. (2001) examined the effect of nisin (10 and 20 µg/g) in combination with 2% NaCl incorporated into raw buffalo meat mince. Growth of inoculated *L. monocytogenes* was significantly inhibited compared with samples not containing nisin.

There have also been investigations into the feasibility of replacing sulfur dioxide with a nisin/organic acid combination in raw pork sausages. Scannell et al. (1997) reported a combination of sodium lactate and nisin reduced the total bacterial count. Jarvis and Burke (1976) reported that spoilage was delayed by a combination of 10 µg/g nisin in combination with 1000 ppm sorbic acid and 2.5% polyphosphate. Luo and Zhu (2000) showed that nisin had a significant effect on the preservation of fresh beef slices and that this was synergistic with sodium lactate.

Cooked Meat Products

Initial studies on nisin use in cooked meat products were targeted at reducing nitrite levels in these foods (Rayman et al., 1981; Calderon et al., 1985; Taylor et al., 1985; Taylor and Somers, 1985). Nitrite must be added for development of the desired pink color. Some prevention of growth of *C. botulinum* and *Clostridium perfringens* was reported, but in general results were inconsistent and extension of shelf life was not reliable. It was suggested that this could be the result of binding of nisin to meat particles, uneven distribution, and poor solubility (de Vuyst and Vandamme, 1994; Henning et al., 1986). It has been suggested that nisin may be inactivated in raw meat by an enzymatic reaction with glutathione (Rose et al., 1999).

Better results have been obtained from nisin use in cooked, cured, chilled sausages and pâté products. This may partly be because nisin can be homogeneously distributed throughout the matrix of these food systems. Reports of nisin efficacy include control of spoilage *Lactobacillus* species, including *L. viridescens*, by 5 µg/g nisin in a frankfurter sausage (Stankiewicz-Berger, 1969). Caserio et al. (1979) described nisin (5 µg/g) control of *C. perfringens* in Italian wurstel sausage. Outgrowth and growth of *B. licheniformis* spores was inhibited by 12.5 µg/g nisin in cooked luncheon meat for 10 days stored at 20°C (Bell and de Lacy, 1986). Growth of the pathogen *L. monocytogenes* was prevented in liver pâté by a combination of nisin, citrate (as chelating agent), and lysozyme (Ter Steeg, 1993; Ter Steeg et al., 1994). *Listeria* control has also been reported for cooked pork tenderloins using a surface treatment of 6.25 µg/g nisin in a modified atmosphere (Fang and Lin, 1994).

The formulation of cooked cured meat products can vary considerably in their composition and levels of fat, moisture, NaCl, nitrite, and phosphate. In a chilled, vacuum-packed bologna sausage product, nisin was more effective against lactic acid bacterial growth in formulations with higher percentage of lean meat (Davies et al., 1999). The type of phosphate was also found to influence both nisin efficacy and bacterial growth. Optimum efficacy was reported for a formulation containing 25% fat and diphosphate as an emulsifier. Significant shelf life extension was obtained with 6.25 to 25 µg/g nisin. A combination of lysozyme, nisin, and EDTA was investigated by Gill and Holley (2000a) in a similar product. The treatment reduced counts of lactic acid bacteria and *B. thermosphacta*. Inhibition of *E. coli* O157 was achieved for 4 weeks in ham sausages. The next step was to explore this combination applied as a surface coating to the sausages using gelatin (Gill and Holley, 2000b). This had an immediate cidal effect against Gram-positive bacteria and inhibited growth during the 4-week storage period. The gel also had a bactericidal effect on *S. Typhimurium*

during storage. Kassaify et al. (2000) have shown good nisin uptake in natural and collagen casings; this could be an alternative approach to the preservation of cooked sausages.

Homemade-style pasteurized chilled meat gravies and sauces are becoming increasingly popular. These products are part of the expanding range of convenience foods offered as a time-saving alternative to the busy consumer. They are often marketed as being fresh, tasty, and containing natural ingredients. Because these foods are not fully retorted, heat-resistant spores survive and psychotrophic *Bacillus* species may grow during chilled storage. Beuchat et al. (1977) reported that nisin at 1 µg/ml inhibited growth of psychotrophic *B. cereus* at 8°C in beef gravy and delayed diarrheal enterotoxin production by vegetative cells and spores.

A 2001 study by Nattress et al. evaluated the combination of lysozyme and nisin to control the meat spoilage bacteria *B. thermosphacta* and *Carnobacterium* in broth, in pork juice extract, and on cores of lean and fat pork. The combination was more effective than either agent used singly. Bell and de Lacey (1987) successfully used a combination of nisin (12.5 µg/g), sorbic acid (0.125%), and monolaurin (0.25% to 0.5%) to control spoilage by *B. licheniformis* as well as the natural spoilage microflora in a pasteurized cure meat product.

FISH AND SHELLFISH APPLICATIONS

It is becoming increasingly apparent that the psychotrophic pathogen *L. monocytogenes* is a very real problem in fresh and lightly preserved seafood products. These products are often not heated before consumption, and some are transported at ambient temperatures. Cold smoked salmon and trout are examples of such products where growth of *Listeria* has been reported; these products are usually vacuum-packed, contain <5% NaCl, and have a pH greater than 5 (Rørvik et al., 1991; Ben Embarek and Huss, 1992; Eklund et al., 1995). Nisin in combination with a carbon dioxide atmosphere showed enhanced effectiveness against *L. monocytogenes* on cold smoked salmon (Nilsson et al., 1997; Szabo and Cahill, 1998). Nisin (100 to 150 µg/g) and 60% sodium lactate synergistically inhibited listeria when injected into rainbow trout before cold smoking (Nykanen et al., 2000). The antimicrobial treatments did not adversely affect the sensory quality of the fish, and they remained acceptable to a sensory panel for 1 week more than untreated samples.

Listeria is also a problem in shellfish. Canned frozen lobster is usually heat processed, but the treatment can be no greater than 60°C for 5 minutes, otherwise product shrinking occurs. This only reduces counts of *L. monocytogenes* by 2 logs, unless nisin is added to the brine at a concentration of 25 µg/ml. This achieves a 5- to 6-log reduction (Budu-Amoaka et al., 1999). The synergy between heat and nisin against *L. monocytogenes* was confirmed in 2000 (Modi et al., 2000). Washing of blue crab (*Callinectes sapidus*) with 2.5 or 5 µg/ml nisin reduced listerial counts by 2 and 3 logs, respectively (Degnan et al., 1994). Another pathogen causing concern in vacuum-packed or modified atmosphere packaging (MAP) packed fish is *C. botulinum*. Toxin production was delayed by 5 days in a study investigating nisin treatment of fresh fish and smoked fish fillets packaged in 100% carbon dioxide and stored at 10°C (Taylor et al., 1990).

BEVERAGES

Fruit Juice

The novel acid-tolerant, endospore-forming Gram-positive bacterium *Alicyclobacillus acidoterrestris* can cause flat sour spoilage of low pH products such as fruit juice (Cerny et al., 1984). Both the vegetative cells and spores of this heat-resistant bacterium are extremely sensitive to nisin (Komitopoulou et al., 1999; Yamazaki et al., 2000), and the presence of nisin during the pasteurization decreases the *D*-value by approximately 40% (Komitopoulou et al., 1999).

Alcoholic Beverages

The realization that nisin can control lactic acid bacteria without affecting fungi inspired researchers at the Brewing Research Foundation in the United Kingdom to investigate the use of nisin for the exclusion of spoilage bacteria in the manufacture of alcoholic beverages. Ogden and coworkers found no detrimental effect of nisin on the performance of the commercial strains of brewing yeast (Ogden and Tubb, 1985; Ogden, 1986; Ogden and Waites, 1986; Ogden et al., 1988). In addition to using nisin during fermentation and after bottling, nisin was used to “wash” the pitching yeast contaminated with lactic acid bacteria (Ogden, 1987). Nisin at 25 µg/ml reduced viability of the contaminant spoilage organisms by 92% after 4 hours of treatment. This method was superior to the traditional acid-washing procedures for lactic acid bacterial inactivation because the nisin treatment left the *Saccharomyces* cultures with improved viability and vitality with unchanged flocculative and fermentative characteristics. Nisin has also been evaluated in fruit brandies and wines (Henning et al., 1986; Radler, 1990a,b; Delves-Broughton, 1990). It is interesting that a more recent study by Chihib et al. (1999) reported that the Gram-negative, anaerobic, beer spoilage bacterium *Pectinatus frisingensis* is nisin-sensitive.

Daeschel et al. (1991) used nisin-resistant mutants of *Leuconostoc oenos* to successfully conduct a pure culture malolactic fermentation in wine with added nisin containing undesirable lactic acid bacteria. Naturally occurring lactic acid bacteria can cause inconsistent malolactate fermentations, and it can spoil the wine. If these organisms are inhibited by nisin, the level of sulfur dioxide added to control bacterial spoilage can be reduced. Residual nisin was detected in the wine 4 months after its addition.

MISCELLANEOUS APPLICATIONS

Crumpets

Crumpets are high-moisture, flour-based products prepared using yeast or a raising agent and cooked on a hot plate. Usually they are toasted or grilled then spread with butter and jam before eating. Crumpets are popular in the United Kingdom, Australia, and New Zealand. Similar products are Scotch pancakes, drop scones, and pikelets. Crumpets have a water activity of 0.95 to 0.97 and are of neutral pH and consequently have a maximum shelf life of 5 days at ambient temperature. The warmer climate of Australia allows the food-poisoning organism *B. cereus* to grow within the crumpets because the mild cooking process will not kill the spores of this organism. During the shelf life the spores can germinate and the bacilli can grow to levels $>10^5$ cfu/g, when toxin production will occur. Outbreaks have resulted (Murrell, 1978; Lee, 1988). Addition of 3.75 µg/g nisin to the batter inhibits *B. cereus* growth during shelf life, keeping bacterial numbers within safe levels (Jenson et al., 1994).

Pasteurized Liquid Egg

Whole liquid egg is pasteurized to ensure destruction of *Salmonella*, but it is not possible to use a more severe heat treatment because this will coagulate the egg. Heat-resistant and spore-forming Gram-positive bacteria may survive. Growth of these organisms is effectively controlled by nisin (Delves-Broughton et al., 1992). Significant shelf life extension and control of psychotropic *B. cereus* can be achieved using nisin at 2.5 to 5.0 µg/g. A nonthermal pasteurization process has been described by Calderon-Miranda et al. (1999) using nisin with a pulsed electric field treatment. A synergistic combination of nisin with high hydrostatic pressure achieving enhanced kill of *L. innocua* and *E. coli* in liquid egg has also been reported (Ponce et al., 2000).

Salad Dressings

High levels of acetic acid are traditionally used in these products to lower the pH and inhibit growth of lactic acid spoilage bacteria. Normally these dressings are between pH 2.9 to 4.4, depending on the acetic acid content. Nisin has proved an effective preservative in dressings formulated at less acidic pH values. These are becoming popular because consumers often prefer their milder taste and mouthfeel, but these products are susceptible to bacterial spoilage. Muriana and Kanach (1995) described effective control of lactic acid bacteria growth by nisin at 2.5 to 5.0 $\mu\text{g/g}$ in buttermilk ranch dressing at pH 4.2.

Fresh Pasteurized Chilled Soups

These pasteurized products are found in the chill cabinet in supermarkets and are proving increasingly popular. The soups have the taste and appearance of a home-cooked dish. Nisin is an effective preservative, which has led to its use in the United States, where it has been allowed for certain manufacturers by the process of self-affirmation. Nisin at a level of 2.5 to 6.25 $\mu\text{g/g}$ controls the growth of spoilage *Bacillus* and extends the shelf life.

Soy and Vegetarian Products

Work in Danisco laboratories has shown that nisin at 1 to 2.5 $\mu\text{g/ml}$ controls spoilage and extends the shelf life of UHT and pasteurized soy milk. The growth of *B. stearothermophilus* spores was prevented by 2.5 $\mu\text{g/ml}$ nisin in soy milk for 7 days at 55°C (Thomas and Delves-Broughton, 2001). UHT treatment can produce a bitter taste in the milk and nisin addition would enable a reduction in the heat process, resulting in a better product with good shelf life (Thomas and Delves-Broughton, 2001). Coconut milk/water can become discolored by UHT processing, but low levels of nisin can protect against spoilage if the heat process is reduced. The use of titanium dioxide as a whitening agent should be avoided in these products if nisin is used. Nisin as a preservative in other soybean products including tofu (nonfermented) and the fermented Japanese product miso has also been investigated (Kato et al., 2001; Schillinger et al., 2001; Thomas and Delves-Broughton, 2001). Fang et al. (1997) reported successful inhibition by nisin of *S. aureus* and *B. cereus* in a dried bean curd product. Nisin together with nisin-resistant lactic acid bacterial starter cultures has also been investigated in fermented vegetable products, including fermented cabbage, sauerkraut, and kimchi (Harris et al., 1992; Breidt et al., 1993; Oh et al., 1994).

Cooked Potato Products

There is potential for nisin use as a preservative in potato cakes, a high-moisture product stored at ambient temperature. The associated spoilage and safety problems are the same as described for crumpets. Potato cakes have a moisture content of approximately 56%, even higher than crumpets, and examination of such products in our laboratories found high levels of *Bacillus* (Thomas and Delves-Broughton, 2001). Cooked mashed potato is another convenience product whose shelf life is compromised by *Bacillus* and *Clostridium* strains that survive the cooking process. Studies in our laboratories have shown effective control of both these organisms at both chill and ambient temperatures and significant shelf life extension by nisin at levels of 6.25 to 25 $\mu\text{g/g}$.

Fresh Pizza

Ham pizza stored in modified atmosphere packaging under 20% carbon dioxide spoils as a result of the growth of lactic acid bacteria and yeasts. Cabo et al. (2001) used a rotatable factorial design to investigate the effects of nisin (as Nisaplin®) and carbon dioxide on the stability of ham pizza

during storage. The combination of carbon dioxide and nisin significantly increased the shelf life of ham pizza by the inhibitory action of the gas against the yeasts and the nisin against the spoilage bacteria. Optimum spoilage prevention was achieved using highly enriched carbon dioxide-enriched atmospheres with 100 mg/kg Nisaplin® (2.5 µg/g nisin) added to the top of the pizza and mixed in the tomato paste.

GENETICS

Nisin production in most strains of *L. lactis* is encoded by large conjugative nisin transposons located on the chromosome that also encode sucrose fermenting ability (Horn et al., 1990; Rauch and de Vos, 1992). Transposons coding for nisin A and nisin Z were found evenly distributed in a study of more than 20 *L. lactis* strains (de Vos et al., 1993). Several of the genes in nisin gene clusters have been sequenced (Engelke et al., 1992a,b; van der Meer et al., 1993; Kuipers et al., 1993; de Vos et al., 1995; Siegers and Entian, 1995). A cluster of 11 contiguous genes in the order *nisABTCIPRKFEG* are involved in nisin biosynthesis (de Ruyter et al., 1996; Siezen et al., 1996). These genes include those that, apart from the structural gene *nisA*, are involved in intracellular modification (*nisBC*), translocation (*nisT*), extracellular proteolytic activation (*nisP*), and regulation of the synthesis (*nisRK*). Genes for two systems coding for nisin immunity (*nisI* and *nisFEG*) are also present in the cluster.

The 57 amino acid nisin A precursor peptide is encoded by *nisA*. It is thought that the 993-amino acid residue *NisB* and the 414-amino acid residue *NisC* are involved in the posttranslational modifications such as the synthesis of thioether amino acids and the α ,-unsaturated amino acids didehydroalanine and didehydrobutyrine. There is as yet no evidence regarding the enzymatic function of these proteins (Kupke and Götz, 1996). *NisB* is located in the cytoplasmic membrane, and it is thought that *NisC* may be located in the cytoplasm (Engelke et al., 1992a; de Vos et al., 1995). Prior to translocation, the presence of the leader C-terminal 23-residue peptide renders the 34-residue propeptide nisin inactive. *NisT* mediates extrusion of the nisin precursor. Once outside the cell the leader peptidase *NisP* acts on the nisin precursor molecule. *NisP* has an N-terminal signal sequence and a C-terminal membrane anchor, indicative of secretion followed by anchoring to the cell membrane (van der Meer et al., 1993).

It is thought that there are two different systems involved in the self-protection nisin immune mechanism, coded for by *nisI* and *nisFEG* (Siegers and Entian, 1995; Saris et al., 1996). The immune mechanism is not fully understood, but it is thought that at least part of the *NisI* immunity protein is attached to the outer side of the cytoplasmic membrane. The proteins *NisE* and *NisF* show strong homology with the ATP-binding cassette transporters that are thought to be involved in nisin extrusion. The gene *nisG* encodes a hydrophobic protein that may act in a similar fashion to the immune proteins described for some colicins (Siegers and Entian, 1995).

Transcription of *nisA* may be autoregulated and may require intact *nisK* and *nisR* genes. The translocated modified nisin molecule can extracellularly induce transcription of the nisin structural gene via signal transduction by the *NisK-NisR* bi-component regulatory system (Kuipers et al., 1995). The *nisF* promoter is also nisin-inducible and subject to the same *nisRK*-dependent control as the *nisA* promoter (de Ruyter et al., 1996). The nisin concentration-dependent transcription of the *nisFEG* genes may be necessary to acquire sufficient immunity to counteract the high nisin concentrations produced by the wild type. The regulation may allow immunity levels to be increased rapidly in the presence of higher nisin concentrations.

The investigations and understanding of nisin biosynthesis have been concurrent with interest in protein engineering strategies aimed at constructing nisin variants with desired properties and also at increasing our understanding of nisin activity. Several mutant nisin species have been produced by site-directed mutagenesis (Dodd et al., 1992; Kuipers et al., 1992; Rollema et al., 1995; Kuipers et al., 1996). These studies have provided evidence, for instance, that nisin activity involves distinct structural features of the nisin molecule.

TOXICITY

The first systematic study of nisin oral toxicity appears to be that of Hara et al. (1962) in Japan. This report has not been widely published and is largely unknown to English readers. They investigated oral administration to rats and kittens and found the median lethal dose (LD₅₀) to be similar to that of common salt — that is, about 7 g/kg. This result confirmed the previous assumption that lactococci and their metabolic products are harmless because nisin-producing lactococci have been isolated from farmhouse cheeses (Chevalier et al., 1957). Because nisin-producing lactococci are present in some cheeses, it is assumed that nisin may occur in milk and cheese as a result of chance contamination. Delves-Broughton (1990) reported a study in which 109 of 251 raw milk samples from around the world contained nisin-producing *L. lactis* subspecies *lactis* populations. Therefore it is probable that people have consumed nisin for a long time without apparent ill effects (Foods Standards Committee, 1959). In a study of nisin toxicity, Frazer et al. (1962) emphasized that this does not prove nisin to be harmless, especially if applied in a different context, but it indicates that at least it has a low toxicity.

The acute toxicity and long-term effects of feeding up to a 1400-fold excess of nisin were studied in Great Britain by Frazer et al. (1962). In rat studies, these workers examined body weight gain, food intake, reproductive performance, survival of young, hematologic picture, mortality rate, organ weight, and gross and microscopic appearance of organs. Special attention was paid to nephrotoxic action. Also, the sensitizing action of nisin was studied in guinea pigs. These researchers observed no consistently significant toxic finding, and it was concluded that nisin was a safe substance for use in food. This is a very safe conclusion because the nisin levels fed to the animals in these and other studies were far in excess of those used in foods. Shtenberg and Ignat'ev (1970) in the Soviet Union extended these studies to nisin alone and in combination with other preservatives, such as sorbic acid. They confirmed the lack of toxicity of nisin. Lipinska (1977) also refers to other studies in the Soviet Union, all of which confirmed the atoxicity of nisin.

To further reassure consumers, a study by Claypool et al. (1966) showed that 10 minutes after consumption of chocolate milk containing nisin, the preservative could not be detected in human saliva. Nisin is rapidly inactivated by digestive enzymes. There has also never been any evidence of any cross resistance occurring to medically important antibiotics (Szybalski, 1953; Carlson and Bauer, 1957; Hossack et al., 1983; Rogers and Hilton, 2001). Mazzotta et al. (2000) have also demonstrated that nisin-resistant *L. monocytogenes* and *C. botulinum* did not become resistant to other preservation factors (NaCl, low pH, sodium nitrite, and potassium sorbate) that could be present in other multiple hurdle systems. In general these strains were more preservative-sensitive to other inhibitors.

LEGAL STATUS

In 1969 the Joint FAO/WHO Expert Committee on Food Additives gave nisin international acceptance as a food preservative (Anonymous, 1969). Nisin and the antifungal agent natamycin remain the only natural antimicrobial compounds allowed as preservatives in foods (Henning et al., 1986). The FAO/WHO Expert Committee recommended that use of nisin as a food “be considered acceptable, the unconditional Average Daily Intake being 0 to 33,000 units/kg of body weight.” The FAO/WHO Codex Committee on milk and milk products recommended standards for acceptance by national governments. For processed cheese, nisin was included as a food additive at 12.5 mg pure nisin per kilogram product.

National legislations vary widely on the use of nisin in foods (Table 7.3). The EU designated food additive number for nisin is E234. Mercosur allows 12.5 µg/g in all types of cheese. The Codex Alimentarius Commission presently allows use of nisin at 12.5 µg/g in processed cheese and processed cheese preparations including spreads. This is at present being reviewed and has a draft list of usages in cereal and starch-based desserts (3 µg/g); clotted cream (10 µg/g); unripened,

TABLE 7.3
International Regulations on Nisin Use

Geographic Area	Food in Which Nisin Is Permitted	Maximum Addition Level (vg/g)
Abu Dhabi	Pasteurized, flavored, and long-life milks; processed cheese; other dairy products; canned foods	No limit
Algeria	Processed cheese	2.5
Argentina	Cheese, processed cheese, requeijao, and ricotta	12.5
Australia ^a	Crumpets, flapjacks, and pikelets (hot plate products)	25
	Cheese, processed cheese, and reduced-fat processed cheese and cheese spreads; processed cheese food; club cheese; blended cheese; canned tomatoes; tomato paste and tomato puree with pH <4.5; canned soups given a botulinum process; beer	No limit
Bahrain	Pasteurized, flavored, and long-life milks; processed cheese; cheese; other dairy products; canned foods	No limit
Bolivia	Permitted additive	No limit
Brazil	Cheese, processed cheese, pasteurized cheese, requeijao, and ricotta	12.5
Bulgaria	Cheese	5
	Ice for storing fish	No limit
Chile	Cheese	12.5
China	Canned foods and plant protein	5
	Dairy and meat produce	12.5
Colombia	Cheese	12.5
Costa Rica	Cheese products	No limit
Croatia	Cheese	12.5
	Mascarpone	10
Cyprus	Cheese, clotted cream, canned vegetables	No limit
Czech Republic	Cheeses	12.5
	Semolina, tapioca and similar puddings	3
Ecuador	All foods	Not specified
Egypt	Processed cheese and processed cheese paste	12.5
Estonia	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
European Union	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
	Mascarpone	10
Gibraltar	Canned foods (pH <4.5 or given botulinum process), cheese, clotted cream	No limit
Guyana	Canned foods, including canned meat, with pH <4.5, or given botulinum process, clotted cream	No limit
Hong Kong	Canned foods, cheese, clotted cream	No limit
Iceland	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
India	Processed cheese and cheese	100
	Coconut water	125
Indonesia	Cheese preparations	12.5
Israel	Cheese (except soft white cheese)	No limit
Jordan	Processed cheese and spreadable processed cheese	12.5

(continued)

TABLE 7.3 (CONTINUED)
International Regulations on Nisin Use

Geographic Area	Food in Which Nisin Is Permitted	Maximum Addition Level (vg/g)
Kuwait	Processed cheese and processed cheese preparations	12.5
Latvia	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
Lithuania	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
Macedonia	Processed cheese	12.5
Malaysia	Cheese, canned foods given a botulinum process	No limit
Malta	Cheese, clotted cream, canned foods	No limit
Mauritius	Canned foods, cheese, clotted cream	No limit
Mexico	Permitted additive	No limit
	Processed cheese	12.5
Montenegro	Processed cheese	12.5
Morocco	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
New Zealand	Processed cheese and cheese food, spreadable processed cheese, and processed cheese spread	12.5
	Beer	5
Panama	Fresh cheeses	No limit
Papua New Guinea	Tomato puree, canned tomato pulp, juice and paste, canned fruit (all with pH <4.5), cheese	No limit
Paraguay	Cheese, processed cheese, requeijao, ricotta	10
Peru	Permitted additive	No limit
Philippines	Processed cheese	100
Poland	Processed cheese	12
Qatar	Milk and milk products	No limit
Romania	Any food	2.5
Russia	Processed cheese	5
	Canned vegetables	2.5
Saudi Arabia	Processed cheese and processed cheese spread, processed cheese with vegetable oils, other foods and dairy products	12.5
		No limit
Serbia	Processed cheese	12.5
Singapore	Cheese, canned foods given a botulinum process	No limit
Slovak Republic	Bakery products, sterilized and soured vegetables, concentrated milk products, desserts, cheese, ready meals, semi-canned products, mayonnaise and its products, sauces, creams, beer	12.5
Slovenia	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca and similar puddings	3
	Mascarpone	10
South Africa	Cheeses, processed or blended cheese including cheese spread, processed cheese preparations, and soft cheese	12.5
Sri Lanka	Permitted additive	No limit
Taiwan	Cheese	125

(continued)

TABLE 7.3 (CONTINUED)
International Regulations on Nisin Use

Geographic Area	Food in Which Nisin Is Permitted	Maximum Addition Level (vg/g)
Thailand	Processed cheese	100
Trinidad and Tobago	Canned foods given a botulinum process, clotted cream, cheese	No limit
Tunisia	Clotted cream	10
	Ripened and processed cheese	12.5
Turkey	Clotted cream	10
	Ripened and processed cheese	12.5
	Semolina, tapioca, and similar puddings	3
Ukraine	Preserved vegetables (green peas, tomatoes, colored cabbage, etc.)	100
	Hard and processed cheese	12.5
Uruguay	Cheese, processed cheese, requeijao, and ricotta	10
United States	Pasteurized and processed cheese spreads	25
	Sauces and nonstandard salad dressings	15
	Pasteurized, chilled soups	5
	Liquid egg products (only allowed with written permission from the U.S. Department of Agriculture)	15
Venezuela	Processed cheese	12.5
Vietnam	Cheese % cheese products	12.5

^a Approval for Australia/New Zealand is being sought for cream products at 10 µg/g; crumpets, flapjacks, and pikelets at 25 µg/g; and GMP for cheese and cheese products; oil emulsions (<80% oil); tomato products pH <4.5; fruit and vegetable preparations; liquid egg products; tomato juice <pH 4.5; beer and related products; dairy and fat-based desserts; dips and snacks; and sauces and toppings, including mayonnaise and salad dressings.

ripened, and whey cheese; cheese analogs (12.5 µg/g); processed cheese (25 µg/g); canned or bottled (pasteurized) vegetables; and ready-to-eat soups and broths (GMP). Further approvals are being sought in Canada (processed cheese, liquid egg, frozen canned lobster), the European Union (liquid egg products), Japan (as an approved additive), Korea (processed cheese), and Thailand (extensions for other uses).

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8 Natamycin

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CONTENTS

Introduction	275
Physical and Chemical Properties	276
Antimicrobial Activity	277
Mode of Action	280
Food Applications	280
Cheese	280
Meat	282
Fruit Juices	283
Miscellaneous Applications	284
Regulatory Status	284
Toxicology	286
Assay	286
References	287

INTRODUCTION

Natamycin has been used for several years as a preservative for the prevention of spoilage resulting from yeast or mold growth associated with certain foods and beverages. Natamycin was first discovered in 1955 in a culture filtrate of *Streptomyces natalensis* isolated from a soil sample in the Natal Province of South Africa, and it is from this region that the name is derived. An earlier name was pimaracin (named after the nearby town of Pietermaritzberg), but, although this name can be found in earlier publications, the World Health Organization (WHO) no longer accepts it. Tenneccin, an antimicrobial isolated from a culture of *Streptomyces chattanoogensis* in 1959 (Burns and Holtman, 1959), was later discovered to be identical to natamycin (Brik, 1981). Natamycin preparations are available commercially under the trade names Natamax™ (Danisco) and Delvolid® (DSM), both of which contain approximately 50% natamycin blended with lactose. Various modified products are also available for specific applications such as surface treatment of cheese and meats (Stark, 1999). Commercial preparations are made by the controlled fermentation in dextrose-based media by selected *Streptomyces* strains. Dried natamycin recovered from the fermentation broth is white to cream colored and has little or no odor or taste; in the crystalline form it is very stable. Optimization of nutrients in the fermentation media for natamycin production by *S. natalensis* in submerged batch culture has been determined (Farid et al., 2000).

Natamycin is particularly useful in foods to prevent the growth of fungi. Fungi grow on foods that have low pH, moisture, and water activity; fungi are also able to grow at low temperature. Some fungi can produce secondary metabolites called mycotoxins that are carcinogenic, teratogenic, hemorrhagic, or dermatitic (Davis and Diener, 1987). Natamycin has been used in the past in human

and veterinary medicine for the topical treatment of fungal and infections, but such use has now been mainly superseded by other antimycotic preparations.

Natamycin has been the subject of several reviews (Raab, 1972; Brik, 1981; Stark, 1999; Thomas and Delves-Broughton, 2001).

PHYSICAL AND CHEMICAL PROPERTIES

Natamycin is a tetraene polyene macrolide with a molecular weight of 665.7 Daltons. The full chemical name is 22-(3-amino-3,6-dideoxy-b-D-manno pyranosol) oxy-1,3,26 trihydroxy-12-methyl-10-oxo-6,11,28-trioxiatri [22.3.1.05.7] o catosa-8, 14,16,18,20-pentanene-25-carboxylic acid. The empirical formula is $C_{33}H_{47}NO_{13}$, and the full structure as elucidated by Ceder (1964) is shown in Figure 8.1. The stereo-structure of natamycin was described by Lancelin and Beau (1990). In its common crystalline form, natamycin exists as a trihydrate. As a dry powder it can be stored for several years with minimal loss of activity. Factors affecting the stability are extreme pH value, light, oxidants, chlorine, and heavy metals. Like many polyene macrolides, natamycin is amphoteric, possessing one acidic and one basic group. Similar compounds include nystatin, rimodacin, lucenocin, tetrin A, tetrin B, and amphotericin (Hamilton-Miller, 1973; Brik, 1981; Thomas and Delves-Broughton, 2001). Natamycin has low solubility in water (approximately 40 $\mu\text{g/ml}$). This low solubility is an advantage for the surface treatment of food because it ensures that the preservative remains on the surface of food where it is needed, instead of migrating into the food. Solubility ranges in other solvents include ($\mu\text{g/ml}$) methanol, 1200; *n*-butanol, 50 to 120; glycerol, 3000; and ethanol, 100 (Brik, 1971; Raab, 1972; Danisco unpublished results). The pH of a 2% suspension of NatamaxTM or Delvolid[®] is 5 to 7.5.

Raab (1972) reports in detail on the effect of pH on the stability of natamycin solutions in phosphate buffers over the pH range 2 to 10. At pH values of 4.5 and 9, natamycin shows good stability at 37°C over a 72-hour period, whereas at extreme pH values of 2 and 10, stability is significantly reduced. Stability at 100°C is optimum at pH 6 and 7 and less optimum at pH levels higher and below. Raab (1967) reported the isoelectric point of natamycin as 6.5.

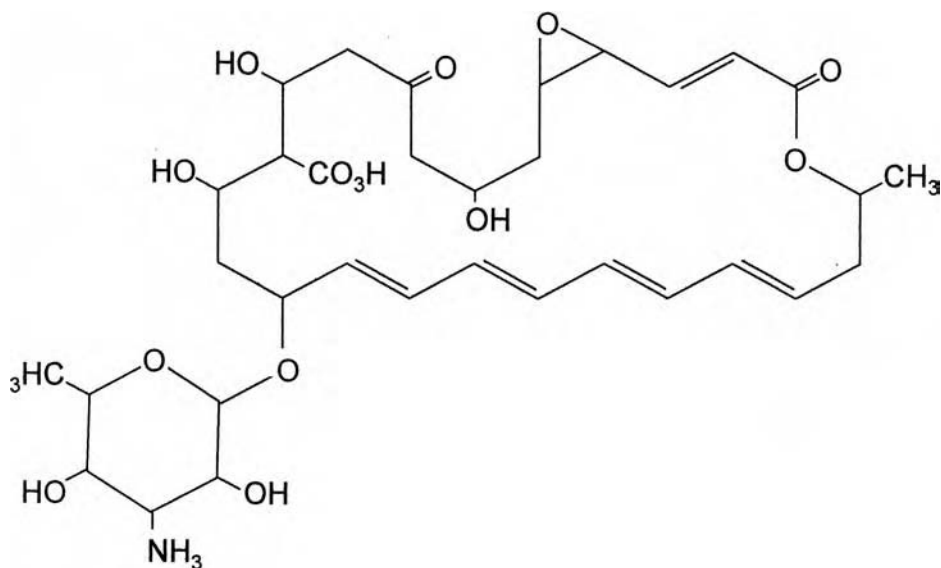


FIGURE 8.1 The structure of natamycin.

Despite the general interest in polyene macrolides, little has been elucidated about the biosynthetic routes and the gene clusters encoding them. Aparicio et al. (1999) cloned a large *S. natalensis* gene cluster involved in the biosynthesis of the polyketide backbone that forms the 26-membered tetraene macrolide ring of natamycin. The biosynthetic cluster extended for about 110 kilobase pairs of contiguous DNA in the genome. A group of small polyketide-functionalizing genes was located between two sets of polyketide synthase genes. Two of the latter genes were fully sequenced. The *pimS0* gene was required for chain initiation, and the *pimS1* gene encoded a giant multi-enzyme involved in 15 activities responsible for the four rounds of chain elongation.

ANTIMICROBIAL ACTIVITY

Natamycin is active against nearly all yeasts and molds but has no effect on bacteria, protozoa, or viruses. Food industries that rely on fermentations by bacteria have found natamycin to be very useful because it does not interfere with fermentation or ripening processes. Most molds are inhibited at concentrations of 0.5 to 6 µg/ml, but some species require 10 to 25 µg/ml (Table 8.1). Most yeasts are inhibited at concentrations from 1.0 to 5.0 µg/ml. (Table 8.2).

Several workers have shown that subinhibitory levels of natamycin can inhibit mycotoxin production. Ray and Bullerman (1982) reported that 10 µg/ml natamycin inhibited aflatoxin B₁ production of *Aspergillus flavus* by 62.0% and eliminated ochratoxin production by *A. ochraceus*. The same level of natamycin inhibited penicillic acid production by *Penicillium cyclopium* by 98.8% and eliminated patulin production of *P. patulum*. The inhibitory effect of natamycin was reported to be greater against mycotoxin production than against mycelial growth. Gourama and Bullerman (1988) studied the effect of natamycin on growth and mycotoxin production (penicillic acid) by *Aspergillus ochraceus* OL24 in yeast extract-sucrose (YES) medium and olive paste. Natamycin at 20 µg/ml delayed onset of growth of *A. ochraceus* by 2, 4, and 16 days in YES broth compared to the control at 15°C, 25°C, and 35°C, respectively. Sporulation of the microorganism was completely inhibited at 15°C by 20 µg/ml natamycin and was delayed 6 days at 25°C and 13 days at 35°C. In terms of mycelial weight increase, 20 µg/ml natamycin was effective in reducing growth by 80% to 100% at all three temperatures. In addition, penicillic acid production by *A. ochraceus* at all temperatures was nearly totally inhibited by 20 µg/ml. Even at 10 µg/ml natamycin, penicillic acid production was inhibited 20% to 90%. Significantly these data were in contrast to those found for potassium sorbate, which, at sublethal concentrations, caused stimulation of penicillic acid production by *A. ochraceus*. In olive paste, natamycin was added at 85, 175, and 350 µg/g. Growth initiation was delayed 1 to 4 days by the three levels of natamycin at 25°C. At 85, 175, and 350 µg/g in olive paste, penicillic acid production was inhibited 70%, 90%, and 96%, respectively. The authors concluded that natamycin could provide protection against fungal growth and mycotoxin formation in olives. Similarly Shahani et al. (1977) showed that the mycotoxin produced by the molds *A. flavus*, *A. ochraceus*, *P. cyclopodium*, and *P. patulum* was inhibited at levels of natamycin that did not inhibit their growth. At the lowest level of 1.0 µg/ml natamycin the growth of the molds was inhibited by 0.3% to 23%, whereas their toxin was inhibited by 25% to 97%. Comparative studies were made to determine effects of commonly used antimycotic agents, sorbate, propionate, and benzoate, and they were found to be far less effective. Rusul and Marth (1988) showed that natamycin at concentrations up to 20 µg/ml in glucose yeast extract medium could delay growth and aflatoxin production by *A. parasiticus* and that effectiveness could be enhanced by combining it with other additives or by using other conditions such as low temperature and pH. Such combined use of additives and growth conditions forms the basis of the hurdle concept of food preservation (Leistner and Gorris, 1995).

TABLE 8.1
Sensitivity of Molds to Natamycin

	MIC ^a (vg/ml)
<i>Byssoschlamys fulva</i> 040021	0.1–1.25
<i>Penicillium candidum</i> S66	
<i>P. chrysogenum</i> S138	
<i>P. commune</i> ABC118	
<i>P. cyclopium</i> S124	
<i>P. nalgiovense</i> S125	
<i>Aspergillus chevalieri</i> 4298	0.1–2.5
<i>A. clavatus</i>	
<i>A. nidulans</i>	
<i>A. ochraceus</i> 4069	
<i>Cladosporium cladosporioides</i>	
<i>Gloeosporium album</i>	
<i>Penicillium chrysogenum</i>	
<i>P. islandicum</i>	
<i>P. verrucosum</i> var. <i>cyclopium</i>	
<i>Sclerotinia fructicola</i>	
<i>Botrytis cinerea</i>	1–25
<i>Aspergillus niger</i> CBS733.88	2.5
<i>A. versicolor</i> 108959	
<i>B. nivea</i> 163642	
<i>Fusarium solani</i> S200	
<i>P. roqueforti</i> S44	
<i>Absidia</i> sp.	4.0–8.0
<i>Acremomium sclerotigenum</i>	
<i>Alternaria</i> sp.	
<i>Aspergillus flavus</i> CBS 3005	
<i>A. flavus</i> BB 67	
<i>A. flavus</i> Madagascar	
<i>A. flavus</i> Port Lamy	
<i>A. niger</i>	
<i>A. versicolor</i>	
<i>Mucor mucedo</i>	
<i>Penicillium digitatum</i>	
<i>P. expansum</i>	
<i>P. notatum</i> 4640	
<i>P. nigricans</i>	
<i>P. viridicatum</i> Westling	
<i>Scopulariopsis asperula</i>	
<i>Aspergillus oryzae</i>	10
<i>Fusarium</i> sp.	
<i>Geotrichum candidum</i>	
<i>Penicillium roqueforti</i> var. <i>punctatum</i> 6018	
<i>Rhizopus oryzae</i> 4758	
<i>P. discolor</i> 547.95	>40
<i>P. discolor</i> 549.95	
<i>P. discolor</i> 551.95	

^a Minimum inhibitory concentration (defined as no growth after 5 days at 25°C; inoculum of ca. 10⁴ spores in centre of agar plate).

Source: Adapted from Klis et al (1959), de Boer and Stolk-Horsthuis (1977), Gist-Brocades Food Ingredients (1991), and previously unpublished data from Danisco.

TABLE 8.2
Sensitivity of Yeasts to Natamycin

	MIC ^a (vg/ml)
<i>Brettanomyces bruxellensis</i>	1.0–2.5
<i>Candida albicans</i>	
<i>C. krusei</i> H66	
<i>C. pseudotropicalis</i> H3	
<i>C. valida</i> H74	
<i>C. vini</i>	
<i>Debaryomyces hansenii</i> H42	
<i>Dekkera bruxellensis</i> CBS2796	
<i>D. bruxellensis</i> CBS4459	
<i>D. bruxellensis</i> CBS6055	
<i>Hanseniasporum uvarum</i> CBS5074	
<i>Hansenula polymorpha</i>	
<i>Pichia membranaefaciens</i> H67	
<i>Rhodotorula mucilaginosa</i> CBS8161	
<i>Saccharomyces (Zygosaccharomyces) bailii</i>	
<i>S. bayanus</i>	
<i>S. bayanus</i> IO18-2007	
<i>S. carlsbergensis</i> CRA6413	
<i>S. cerevisiae</i> ATCC9763	
<i>S. cerevisiae</i> CRA124	
<i>S. cerevisiae</i> H78	
<i>S. cerevisiae</i> 8021	
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	
<i>S. exiguus</i>	
<i>S. ludwigii</i> 0339	
<i>Torulopsis candida</i>	
<i>Z. bailii</i> CRA229	
<i>Z. rouxii</i> CBS1640	
<i>Candida guilliermondii</i>	3.0–10.0
<i>C. kefyr</i> H2	
<i>C. paralopsiosis</i> NCYC458	
<i>C. utilis</i> H41	
<i>Kloeckera apiculata</i>	
<i>Kluyveromyces lactis</i> H17	
<i>Rhodotorula gracilis</i>	
<i>Saccharomyces cerevisiae</i>	
<i>S. cerevisiae</i> var. <i>paradoxus</i> H103	
<i>S. exiguus</i> Rees CBS1514	
<i>S. florentinus</i> H79	
<i>S. unisporus</i> H104	
<i>S. (Zygosaccharomyces) rouxii</i> 0562	
<i>S. sake</i> 0305	
<i>Torulopsis lactis-condensi</i>	
<i>Torulasporea rosei</i>	
<i>Zygosaccharomyces barkerii</i>	

^a Minimum inhibitory concentration (defined as inhibition of growth for 14 days at 25°C; inoculum level at ca. 10³ cfu/ml).

Source: Adapted from Klis et al (1959), Gist-Brocades Food Ingredients (1991), and previously unpublished data from Danisco.

MODE OF ACTION

Polyene macrolides such as natamycin complex with sterols, notably ergosterol, which are important in fungal cell membranes. The irreversible binding of natamycin to ergosterol disrupts the cell membrane, increasing the cell permeability, leading to cell death. Ergosterol is absent from the cell membranes of viruses, bacteria, and protozoa, which explains why these microbes are resistant to natamycin (Hamilton-Miller, 1974). Although inhibition of glycolysis and respiration of polyene macrolides can be demonstrated, these are considered secondary to membrane effects. Ziogas et al. (1993) studied 17 natamycin mutants of *A. nidulans*. Some mutants contained no ergosterol; others had reduced levels of the compound compared to the wild type. Ergosterol-deficient mutants were most resistant to natamycin (approximately 14 to 16 µg/ml) compared to the wild type (around 2 µg/ml) but grew at much slower rates. Some wild-type fungi, presumably because the ergosterol content of their membranes is low, have reduced sensitivity to natamycin. Examples are *P. discolor*, *Verticillium cinnabarinum*, and *Botrytis cinerea*.

De Boer and Stolk-Horsthuis (1977) investigated the potential for development of resistance to natamycin among fungi. They found no evidence of resistant fungi in cheese warehouses in which natamycin had been used for various periods, up to several years. They also attempted to induce tolerance in 26 strains of fungi by transferring each culture 25 to 31 times in media containing concentrations of natamycin equal to and higher than the minimum inhibitory concentration (MIC; defined as the lowest concentration that prevented growth after 4 days and 25°C). The MIC following multiple transfers increased in only 8 of 26 strains and only by a maximum of 4 µg/ml. They concluded that the lack of resistance was the result of the strongly fungicidal activity of the compound, along with its environmental instability. Stark (1999) attributes the lack of reported natamycin resistance to possibly result from the fact that natamycin occurs as micelles in solution. Thus when a cell comes into contact with the preservative, it encounters a high and probably lethal dose of the antimycotic.

FOOD APPLICATIONS

Natamycin is usually applied as a surface application, particularly for treatment of the surfaces of hard cheese and dry or ripened sausages such as salami. Other applications such as yogurt, wine, and fruit juice incorporate the natamycin into the food matrix. Table 8.3 outlines various applications of natamycin, method of application, and recommended dosage.

CHEESE

Surface growth of mold on cheese can be a major factor limiting the shelf life of cheeses. As well as being unsightly, there is a danger of mycotoxin production by the mold. Many cheeses are ripened or matured for several months in ripening rooms at temperatures in the range of 10°C to 12°C, and during this period they are very susceptible to mold colonization and surface growth. There are numerous ways of applying natamycin preparations. As a surface treatment natamycin can be applied by spray, painting, or immersion or by adding to a plastic coating then dipping or painting the cheese. Often many such applications are given over a period of time to ensure complete protection. When applied in a polyvinyl acetate (PVA) coating for ripening, the coating can be easily removed prior to retail so the consumer receives a clean yet mature cheese. When natamycin is added by immersion, it is recommended that, to prevent bacterial growth, 10% salt is added to the solution. Natamycin when applied as dipping solutions or sprays will tend to settle out unless stirred or agitated. Modified formulations are available that contain food-grade thickening agents that both prevent settling of the natamycin and improve adhesion to the surface of the product (Stark, 1999). Similarly blends are available that are combined with anticaking agents such as powdered cellulose that can be used in the production of shredded cheese. A novel method of

TABLE 8.3
Applications of Natamycin, with Suggested Dosage Levels and Methods of Application

Food Application	Suggested Natamycin Dosage Levels, ppm	Method
Hard/semi-hard cheese	1250–2000	Surface treatment by spray or immersion
	500	Direct addition to coating emulsion
Meat products: dry sausage	1250–2000	Surface treatment by spray or immersion
Yogurt	5–10	Direct addition to yogurt mix
Bakery products	1250–2000	Surface treatment by spray
Tomato purée/paste	7.5	Direct addition during mixing
Fruit juice	2.5–10	Direct addition
Wine	30–40	Direct addition to stop fermentation
	3–10	Added after bottling to prevent yeast/mold growth

Note: NB: Commercial preparations (Natamax™ and Delvocid®) contain 50% active natamycin.

Source: Thomas & Delves-Broughton (2001).

TABLE 8.4
U.S. Standardized Cheeses that Provide for the Use of Natamycin

Asiago, old	Hard grated cheeses
Asiago, medium	Mozzarella, scamorza
Asiago, fresh, soft	Mozzarella, scamorza, low moisture
Blue	Mozzarella, scamorza, part skim
Caciocavallo	Mozzarella, scamorza, low moisture, part skim
Cheddar	Parmesan, reggiano
Cheddar, low sodium	Provolone
Edam	Romano
Gouda	Samsøe
Gruyère	Swiss for manufacture
Hard cheeses	Swiss, Emmentaler

Source: From Gist-Brocades Food Ingredients (1991).

applying natamycin to the surface of cheese by electrostatic coating has been proposed (Elayedath and Barringer, 2001).

Natamycin is approved by the U.S. Food and Drug Administration (FDA) for use on any standardized cheese for which antimicrobials are allowed (Table 8.4). In the European Union (EU) the maximum level of natamycin permitted on the surface of hard, semi-hard, and semi-soft cheese is 1 mg/square decimeter surface, and natamycin should not be present at a depth greater than 5 mm. Numerous studies have confirmed the efficacy of natamycin as a surface treatment of cheeses, often comparing it to sorbate. Pugazhenti et al. (1999) compared natamycin and potassium sorbate control of *P. citrinum*, a mold that produces the mycotoxin citrinin. Isolates from Swiss cheese samples were inoculated onto the cheese surface. Cheese samples were either left untreated or surface treated with 5 and 10 µg/g natamycin or 500 and 1000 µg/g potassium sorbate. The cheeses were stored at 25°C for 21 days and then analyzed for counts of the fungus. Counts were highest in the control cheeses and lowest in the 10 µg/g natamycin-treated cheese. The lower level of natamycin tested (5 µg/g) was more effective than the highest level of potassium sorbate. A study by de Ruig and van den Berg (1985) demonstrated further advantages of natamycin over sorbate

for the control of mold growth on cheese surfaces. They reported that sorbate, unlike natamycin, caused adverse effects on both the interior and surface of the cheese. Sensory evaluation found sorbate reduced the quality of the cheese, particularly under the rind, and this was most obvious for young cheese. Inner portions of the cheese were also adversely affected by the migration of the sorbate. Natamycin had no adverse effect on the rind or the flavor of the cheese. Engel et al. (1983), using high-performance liquid chromatography (HPLC), showed that natamycin does not penetrate far into the cheese and therefore has less effect on the taste of the cheese and remains predominantly on the surface where mold contamination occurs and needs to be prevented. The depth of penetration of natamycin into the cheese was found to depend on the initial natamycin concentration, storage time, and cheese type. Experiments were carried out at 15°C.

Nilson et al. (1975) determined the effect of natamycin in comparison with mycostatin, on the shelf life of cottage cheese stored at 4.4°C, 10.0°C, or 15.6°C. The compounds were added through curd wash water or in the cheese dressing. The cottage cheese was inoculated with *A. niger* or *Saccharomyces cerevisiae* or was uninoculated. Natamycin (100 µg/ml) added in the wash water was effective in increasing the days to spoilage of uninoculated cottage cheese by 13.6, 7.7, and 6.3 days over the control when stored at 4.4°C, 10.0°C, and 15.6°C, respectively. Cottage cheese inoculated with *A. niger* stored at the same temperatures had increased days to spoilage of 12.7, 6.0, and 4.3 days and samples inoculated with *S. cerevisiae* had increased shelf life of 10.3, 6.3, and 3.7 days, respectively. Adding natamycin to the cottage cheese dressing was even more effective in extending shelf life. At 4.4°C, 10.0°C, and 15.6°C, the inoculated and uninoculated cottage cheese had increased days to spoilage ranges of 20.4 to 26.7, 9.7 to 12.3, and 2.6 to 5.0, respectively. Natamycin was found to be slightly more effective than mycostatin. *A. niger* was found to be the most sensitive of the two microorganisms used in the inoculation studies. Lück and Cheeseman (1978) found that 500 or 1000 µg/ml concentration of natamycin delayed mold growth on cheese for up to 6 months but did not prevent it completely. Neviani et al. (1981) treated Italian Caciotta cheese by immersion in 2000 µg/ml natamycin and found it effective in controlling mold growth, with no effect on the population of desired ripening surface bacteria that produce the red color of Caciotta cheese. Verma et al. (1988) evaluated the effectiveness of natamycin against sorbic acid, benzoic acid, and nystatin in inoculated and uninoculated butter and cheese. All four preservatives were effective in reducing fungal growth on uninoculated butter and cheese samples stored 30 days at 7°C. Maximum effect was shown by the antifungal agent nystatin, followed by natamycin. Lodi et al. (1989) found that natamycin was effective in preserving seven types of Italian cheeses with no detrimental effect on ripening. The antimycotic has been used to some extent as a butter preservative by being applied to the wrapper in small concentrations (Anonymous, 1991). Natamycin has been shown to be effective against thread mold *Phoma glomerata*, which causes infections of hard cheeses (Fente-Sampayo et al. 1995; Basilico et al. 2001). Morris and Castberg (1980) used a 1000 µg/ml natamycin dip in a 10% to 15% brine to control surface mold of blue cheese without affecting the desired internal mold growth.

Natamax is used to treat shredded cheese, particularly in the United States (Berry, 1999). A natamycin suspension of 1500 µg/ml, finely sprayed onto 1 metric ton of shredded cheese using a tumbler and a commercial spray system, will provide protection against yeast and mold spoilage in modified atmosphere or vacuum-packed packs of shredded cheese that lose their modified atmosphere or vacuum as a result of pack damage.

MEAT

Natamycin is often used in Europe to treat the surface of dry fermented sausages and has also been used to treat the surface of cooked hams. EU regulations for meat are similar to those for cheese. The maximum level of natamycin allowed on the surface of dry cured sausages is 1 mg/square decimeter surface, with none present at a depth greater than 5 mm.

Holley (1986) compared the efficacy of sorbate and natamycin on the surface mold and ripening of Italian dry salami. Although complete control was not achieved, a dip of 2000 µg/ml immediately after stuffing the casings helped minimize the development of surface mold. Natamycin spray (2×1000 µg/ml) was as good or better than 2500 µg/ml sorbate. The sorbate treatments proved unsatisfactory because of its antibacterial activity, inhibiting the bacteria responsible for the salami-ripening process. Baldini et al. (1979) sprayed Mortadella sausages, salami, and raw hams with different aqueous solutions of natamycin (1000 to 5000 µg/ml) during maturation of the meats at the beginning of storage. For all the products, this treatment effectively prevented surface mold growth when examined in normal conditions of preparation and storage. The preservative did not cause any change to the typical microflora of the salami (the desired growth that contributes to the flavor), nor did it generate off-flavors in the ham. For most samples natamycin was undetectable at the end of the aging and storage period.

Cattaneo et al. (1978) also investigated the protective effects of natamycin spraying and immersion of similar meat products (salami, cured beef) and found the preservative gave good protection against mold growth. Protection was achieved by immersing the sausage casings in the preservative. Natamycin content was higher in natural compared with synthetic casings. Moerman (1972) carried out similar studies with sausages (Snijworst), applying the natamycin either during brining, by dipping or spraying with 50 to 1000 µg/g before or after smoking, or preliminary soaking of the casings (natural, protein, or fiber). Sausages were stored for 5 weeks and inspected for mold formation weekly. With the natural casings, little or no mold growth was observed after 4 weeks, regardless of the method of application. With the protein-based casings, similar results were obtained except with the presoaked casings, where considerable mold growth occurred. With the cellulose-based casings, results were only satisfactory on spraying with 1000 µg/ml natamycin. For practical purposes, spraying with 1000 to 2000 µg/ml was recommended. De Boer et al. (1979) showed that molds and yeasts had comparable natamycin sensitivities irrespective of the natamycin content of the sausages from which they were isolated. This further confirms that development of resistance to natamycin is not a problem.

FRUIT JUICES

Natamycin has been shown to be an effective preservative in both unpasteurized and pasteurized fruit juices, preventing the growth of yeasts and molds. Thomas and Delves-Broughton (2001) investigated the efficacy of natamycin in preventing visible spoilage (surface growth or gas production) of a wide range of yeasts and molds in apple, orange, and pineapple juices. Incubation was at 25°C for the organisms except for the *Byssochlamys* species; these were incubated at their optimum temperature of 37°C. *Byssochlamys* species are often associated with heat-processed foods and beverages because they produce spores that have high heat resistance that can survive the pasteurization processes typically used in fruit juice processing (Pitt and Hocking, 1999). Results for pineapple juice are shown in Table 8.5. More or less similar results were achieved in apple and orange juice. Unpublished data indicate that pasteurization of orange fruit juice at 80°C for 10 minutes results in 70% retention of natamycin.

Shirk and Clark (1963) found that a natamycin addition of 20 µg/g had an immediate reduction effect on yeast and mold counts in orange juice inoculated with *S. cerevisiae* as well as samples that were not inoculated. After 1 week of storage at 2.5°C to 4°C, yeast and mold counts were undetectable and the sample remained unspoiled for the 8-week duration of the test. Control samples not containing natamycin were spoiled within 1 week. A second study then compared the effectiveness of natamycin to sorbic acid. In this study juice inoculated with natural contaminants spoiled after 1 week of storage, whereas levels as low as 1.25 µg/g of natamycin prevented spoilage during the 8 weeks. In comparison, levels as high as 1000 µg/g of sorbic acid were needed to retard yeast growth. The sorbic acid imparted an unpalatable flavor to the fruit juice; natamycin did not.

TABLE 8.5
Natamycin Control of Yeast and Mold Growth in Pineapple Juice

Organism	Days Until Visible Spoilage at Each Natamycin Concentration (ppm)						
	0	1.25	2.5	5	10	20	40
<i>Debaryomyces hansenii</i> H42	3	72	>72	>72	>72	>72	>72
<i>Pichia membranaefaciens</i> H67	3	11	>72	>72	>72	>72	>72
<i>Saccharomyces carlsbergensis</i> ATCC6413	2	8	8	8	>63	>63	>63
<i>Saccharomyces cerevisiae</i> ATCC9763	2	9	>63	>63	>63	>63	>63
<i>S. cerevisiae</i> H78	2	16	16	35	35	>72	>72
<i>S. cerevisiae</i> CRA124	2	14	29	35	35	>72	>72
<i>S. cerevisiae</i> var. <i>paradoxus</i> H103	2	7	7	7	>72	>72	>72
<i>Zygosaccharomyces bailii</i> CRA229	3	8	18	66	66	>72	>72
<i>Penicillium roqueforti</i> S44	2	3	3	6	61	>61	>61
<i>Byssoschlamys fulva</i> 040021	6	6	8	>63	>63	>63	>63
<i>B. nivea</i> 163642	6	6	6	13	13	17	23
<i>B. fulva</i> TSD 0.0796	6	6	6	>63	>63	>63	>63

Source: Danisco, previously unpublished.

MISCELLANEOUS APPLICATIONS

Promising applications of natamycin include yogurt and bakery products. Thomas and Delves-Broughton (2001) indicate that a level of 5 to 10 $\mu\text{g/g}$ natamycin is effective in preventing the growth of yeasts in yogurt. Such use of natamycin is approved and widely used in South Africa. There is no detrimental effect on the yogurt starter cultures, and the natamycin can be added prior to the addition of the starter cultures or with the fruit preparation after the fermentation in the manufacture of fruit-flavored yogurts.

Natamycin has also been found to have potential as a spray on bakery products. Extension of shelf life of tortillas sprayed with natamycin to achieve surface concentrations of 1 and 5 $\mu\text{g/cm}^2$ resulted in increased protection against mold growth. Ticha (1975) found that a level of 100 $\mu\text{g/g}$ natamycin effectively controlled the growth of 5 molds including *A. flavus* isolated from bakery products.

Another potential application is olives. Contamination of these fermented products with toxigenic molds presents a potential health risk as well as an economic problem, especially in Mediterranean countries where olives are popular. In a study investigating an implicated mold, *A. ochraceus*, Gourama and Bullerman (1988) found that natamycin was effective at controlling fungal growth and mycotoxin production on olives and recommended this as a practical solution to the problem.

Natamycin is used in the wine industry in South Africa for the production of sweet wines. Natamycin is added before the fermentation is complete to arrest the fermentation of the yeast; natamycin is also used as a preservative to prevent yeast or mold spoilage. Gerbaux et al. (2000) showed that addition of 40 $\mu\text{g/ml}$ was effective in controlling *Brettanomyces* yeast infections of the French pinot noir red wine.

Although not a food usage, natamycin has been proposed as a selective antifungal agent in microbiological media (Pedersen, 1992; Johansson et al., 1995; Edelstein and Edelstein, 1996).

REGULATORY STATUS

The acceptable daily intake allowed for natamycin (Food and Agriculture Organization Expert Committee on Food Additives) is 0.3 mg/kg body weight/day (Smith and Moss, 1985). Because

natamycin is mainly used for surface treatments the average daily intake is not reached, even by consuming large quantities of treated products. Table 8.6 summarizes the worldwide authorization of natamycin. Details vary from country to country, and one is advised to check the situation in a particular country.

TABLE 8.6
Worldwide Authorization of Natamycin

Country	Codes	Country	Codes
Algeria	A	Lithuania	A
Argentina ^a	AB	Luxembourg ^b	AB
Australia	AB	Mauritius	AB
Austria ^b	AB	Mexico	A
Bahrain	A	Morocco	AB
Belgium ^b	AB	Netherlands ^b	AB
Brazil ^a	AB	New Zealand	AB
Bulgaria	A	Norway	AB
Canada	A	Oman	P
Chile	A	Poland	AB
China	ABCD	Portugal ^b	AB
Colombia	A	Paraguay ^a	A
Croatia	AB	Qatar	P
Cyprus	A	Saudi Arabia	P
Czech Republic	AB	Singapore	A
Denmark ^b	AB	Slovak Republic	AB
Ecuador	A	Slovenia	AB
Egypt	A	Spain ^b	AB
Eire ^b	AB	South Africa	ABDEFGHJK
Estonia	AB	Sweden ^b	AB
Finland ^b	AB	Switzerland	AB
France ^b	AB	Syria	P
Germany ^b	AB	Taiwan	AB
Greece ^b	AB	Tunisia	AB
Hungary	AB	Turkey	AB
Iceland	A	Ukraine	A
India	A	U.A.E.	P
Italy ^b	AB	U.K. ^b	AB
Jordan	P	U.S.	A
Kuwait	P	Uruguay ^a	A
Latvia	AB	Venezuela	A
Lebanon	P	Yemen Republic	P

Note: A = surface treatment of specified cheese, cheese rinds (shredded cheese in United States only); B = surface treatment of specified processed meats; C = surface treatment of certain baked goods; D = fruit juice; E = wine; F = fish products; G = yogurt; H = canned food; I = sour cream; J = cream cheese; K = cottage cheese; P = permitted additive.

^a Mercosur country.

^b European Union country.

TABLE 8.7
Acute Toxicity of Various Polyene Antimicrobials by Different Routes in Mice or Rats

	LD ₅₀ (mg/kg)			
	Intravenous	Subcutaneous	Intraperitoneal	Oral
Amphotericin B	4–6.6	—	28.0	>8000
Candicidin	—	160–280	2.1–7.0	90–400
Natamycin	5–10	5000 ^a	250 ^a	1500 ^a
Nystatin	3	24	8–14	>3500

^a Rats.

Source: Adapted from Hamilton-Miller (1973).

TOXICOLOGY

The intravenous route is the path by which polyene macrolide antimicrobials are most toxic and oral administration is least toxic (Table 8.7). There is apparently no absorption of up to 500 mg/day natamycin from the human intestinal tract after 7 days of administration (Brik, 1981). Levinskas et al. (1966) carried out a study to determine the acute and chronic toxicity of natamycin. The single oral dose LD₅₀ for natamycin in the male rat was found to be 2.73 g/kg (1.99 to 3.73 g/kg) and 4.67 g/kg (3.0 to 7.23 g/kg) for female rats. The oral LD₅₀ for fasted male albino rabbits was 1.42 g/kg (0.46 to 4.39 g/kg). The single dermal dose LD₅₀ was estimated at >1.25 g/kg. For rats, no signs of toxicity occurred after large single doses and no gross lesions could be detected related to natamycin ingestion. Rabbits that died had congested and hemorrhagic gastric mucosa. In a 3-month feeding study with rats, Levinskas et al. (1966) found that animals fed 8000 ppm natamycin had body weights that averaged 54% to 67% of the control group (no natamycin). At 2000 ppm, animal weight averaged 85% of the control group. Natamycin had no apparent effect on body organs nor did it produce any lesions. Oral administration of natamycin to rats for 2 years at up to 1000 ppm did not have an effect on survival of the animals (Levinskas et al., 1966). The diet containing natamycin was judged to have had no adverse effects on food utilization, reproductive performance, neoplasms, or other lesions above that encountered in controls. Dogs were fed diets containing 125, 250, or 500 ppm natamycin for 2 years (Levinskas et al., 1966). Oral administration of 500 ppm resulted in a slight decrease in body weight. No significant hematologic abnormalities nor significant lesions could be attributed to the consumption of natamycin (Levinskas et al., 1966). Nausea, vomiting, and diarrhea in humans given natamycin orally in doses exceeding 1000 mg/day (Brik, 1981) and vomiting and diarrhea in dogs fed 5000 ppm natamycin (Levinskas et al., 1966) have been reported.

ASSAY

Shirk et al (1962) developed a bioassay for natamycin using *S. cerevisiae* as an indicator organism. The standard curve was linear between 0.5 and 15 µg/ml. Ultraviolet spectra maxima for natamycin are 220, 290, 303, and 318 nm and the minima are 250, 295.5, and 311 nm (Gist Brocades, 1991). De Ruig et al. (1987) developed a method for detection of natamycin in cheese and cheese rind by extraction with methanol and ultraviolet spectrometric detection at 311 nm (minima), 317 nm (maxima), and 329 nm or liquid chromatographic separation followed by detection at 303 nm (maxima). In a subsequent collaborative study, De Ruig (1987) reported that both methods gave reasonable to good results for levels of natamycin down to 15 mg/kg (0.9 mg/dm² surface area). Riedl et al. (1984) also reported using methanol extraction of natamycin on cheese and ultraviolet

spectrometry to detect concentrations of 2.5 mg/kg natamycin in cheese. Fletouris et al. (1995) developed a rapid spectrophotometric method for assay of nisin in cheese and cheese rind. Quantitation and detection limits were estimated at 0.5 and 0.25 $\mu\text{g/g}$, respectively.

HPLC assay methods for natamycin have been developed by a number of researchers (Engel et al., 1983; Tuinstra and Traag, 1982; Guldborg, 1984). Tuinstra and Traag (1982) developed a procedure in which cheese was extracted with methanol and natamycin separated by HPLC. The limit of determination of the procedure was 0.05 mg/kg, and recovery at 1 mg/kg cheese was >90%. Luf and Brandl (1986) extracted cheese samples with methanol and subjected extracts to gradient HPLC with ultraviolet detection in a mixture of acetonitrile/acetate buffer (pH 4.4). Using this method, they were able to detect 0.1 $\mu\text{g/ml}$ natamycin, which corresponded to 0.5 ppm natamycin at the cheese surface. Maruyama et al. (1988) extracted surface and inner area samples of cheese with acetonitrile/phosphate buffer and quantified natamycin using reversed-phase HPLC. Minimum detection level for natamycin was 0.2 $\mu\text{g/g}$ cheese, and recovery of >97.5% was achieved when cheese spiked with 8 μg natamycin/gram of natamycin-free cheese was analyzed. In 1990 an enzyme immunoassay for detection of natamycin in cheese was described by Maertlbauer et al. The assay involved a methanol extraction of the cheese, followed by detection of natamycin using an anti-natamycin antibody from rabbits. The detection limit was 0.005 mg/dm² with a recovery of 76% to 84%. Cross-reaction with related antimicrobials (amphotericin B and nystatin) was <0.001%. Capitán-Vallvey et al. (2000) describe rapid ultraviolet spectrophotometric and liquid chromatographic methods for the detection of natamycin in lactoserum matrix. The methods involve protein precipitation followed by centrifugation and no clean-up stage is necessary. Detection and quantitation limits were 0.07 and 0.23 $\mu\text{g/g}$, respectively, for the ultraviolet spectrophotometric method and 0.1 and 0.32 $\mu\text{g/g}$ for the liquid chromatographic method.

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9 Parabens

P. Michael Davidson

CONTENTS

Chemical and Physical Properties	291
Antimicrobial Activity	293
Bacteria	293
Fungi	296
Comparisons to Other Preservatives.....	297
Mechanism of Action.....	297
Regulatory Status	298
Applications	298
Toxicology.....	299
Assay	300
References	301

Phenolic compounds have been used as antimicrobial or antiseptic compounds since 1867 with the introduction of “carbolic acid” by Joseph Lister to sanitize equipment and in surgical procedures. Although the use of phenol has declined over the years as a result of its high toxicity and relatively low antimicrobial activity, other phenolic compounds have been introduced for use as antimicrobials in foods, pharmaceuticals, and cosmetics. Phenolic compounds, important or potentially important in foods as antimicrobials, may be categorized as those currently approved for use in foods (alkyl esters of *p*-hydroxybenzoic acid) and those that occur naturally in foods or that are added to foods through processing (phenol to complex polyphenolics). Information on naturally occurring phenolic compounds or phytophenolic compounds, formerly covered in this chapter in the first and second editions of this title, may now be found in Chapter 14, “Naturally Occurring Compounds — Plant Sources.” This chapter will focus exclusively on alkyl esters of *para*-hydroxybenzoic acid or the parabens.

In most countries, the methyl and propyl esters of *p*-hydroxybenzoic acid (parabens) are allowed for direct addition to foods as antimicrobials. The following sections will review various characteristics of these compounds along with the ethyl, butyl, and heptyl esters that are approved for use in foods by some countries.

CHEMICAL AND PHYSICAL PROPERTIES

Parabens have the general structure shown in Figure 9.1. The molecular weights of the various esters are as follows: methyl (Chemical Abstracts Service (C.A.S.) 99-76-3), 152.15; ethyl (C.A.S. 120-47-8), 166.18; propyl (C.A.S. 94-13-3), 180.21; butyl (C.A.S. 94-26-8), 194.23; and heptyl, 236.21. Solubility data for these compounds are shown in Table 9.1. As might be expected, water solubility is inversely related to alkyl chain length.

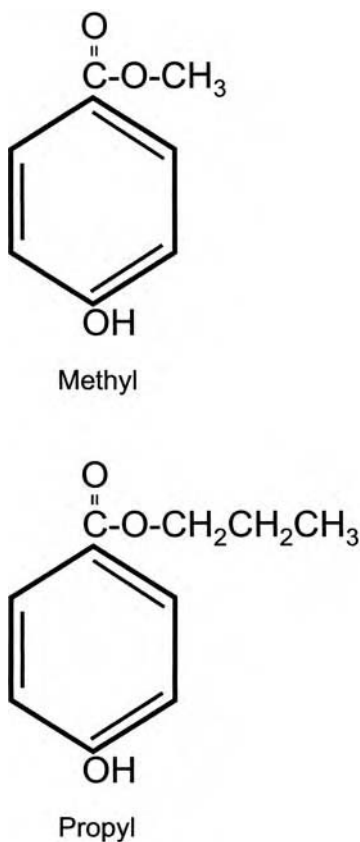


FIGURE 9.1 Structure of the methyl and propyl esters of p-hydroxybenzoic acid.

TABLE 9.1
Solubility of the Parabens in Various Solvents

Solvent	Temperature ^a	Solubility (g/100 g)				
		Methyl	Ethyl	Propyl	Butyl	Heptyl
Water	25°C	0.25	0.17	0.05	0.02	1.5 mg
	10°C	0.20	0.07	0.025	0.005	— ^b
	80°C	2.0	0.86	0.30	0.15	—
Ethanol	25°C	52.0	70.0	95.0	210.0	—
	50% (25°C)	18.0	—	18.0	—	—
	10% (25°C)	0.5	—	0.1	—	—
Propylene glycol	25°C	22.0	25.0	26.0	110.0	—
	50% (25°C)	2.7	—	0.9	—	—
	10% (25°C)	0.3	—	0.06	—	—
Olive oil	25°C	2.9	3.0	5.2	9.9	—
Peanut oil	25°C	0.5	1.0	1.4	5.0	—

^a Solubilities were determined at the temperatures specified; all solvents were pure, except where percentages are shown, which indicates percentage in water.

^b Not reported.

Sources: Aalto et al. (1953); Lück and Jager (1997).

Parabens are stable in air and are resistant to cold and heat, including steam sterilization. Aalto et al. (1953) detected no hydrolysis in solutions of parabens buffered to pH 3.0 and 6.0 and heated at 120°C for 30 minutes. At pH 8.0, 6% hydrolysis was detected under the same conditions. Solutions of the parabens buffered to pH 3.0, 6.0, and 8.0 remained unchanged during storage at 25°C for 6 weeks (Aalto et al., 1953).

Parabens are marketed as ivory to white powders. The compounds are all odorless, except for methyl paraben, which has a faint characteristic odor (Aalto et al., 1953, Chichester and Tanner, 1972).

ANTIMICROBIAL ACTIVITY

The first reports on the antimicrobial activity of the parabens came from Sabalitschka and coworkers in the early 1920s (Prindle, 1983). Esterification of the carboxyl group of benzoic acid allows the molecule to remain undissociated up to pH 8.5 versus normal dissociation of benzoic acid at pH 5.0 (Busta and Foegeding, 1983). Whereas the pH optimum for antimicrobial activity of benzoic acid is 2.5 to 4.0, the parabens are effective at pH 3 to 8 (Aalto et al., 1953; Chichester and Tanner, 1972).

BACTERIA

The antimicrobial activity of parabens has been evaluated against a wide variety of Gram-negative and Gram-positive food-related bacteria (Table 9.2). It should be noted that many inhibition studies were done with different strains of bacteria, incubation conditions (pH, time, temperature), media, assay techniques, and data analyses. Because of these differences, it is difficult to compare results of different studies, except in relative terms.

From the results of the minimum inhibitory concentration (MIC) studies shown in Table 9.2, it can be seen that, as the alkyl chain length of the parabens increases, inhibitory activity generally increases. Increasing activity with decreasing polarity is more evident against Gram-positive than against Gram-negative bacteria. Gram-positive bacteria are generally more susceptible to non-polar phenolic compounds than Gram-negative bacteria. Both Eklund (1980) and Freese et al. (1973) stated that Gram-negative bacteria were, most likely, resistant to the parabens owing to a screening effect by the cell wall lipopolysaccharide layer. Fukahori et al. (1996) studied the relationship between uptake and the antimicrobial activity of the methyl, ethyl, propyl, and butyl esters of p-hydroxybenzoic acid using *Escherichia coli*. They reported that the uptake of the parabens was logarithmically proportional to the alkyl chain length from methyl to butyl. However, free energy change measurements indicated that the transfer of the compounds also involved hydrophilic interactions. In addition, they found that the concentration of parabens necessary for antimicrobial activity decreased in logarithmic relationship to alkyl chain length. They concluded that the antibacterial activity of parabens is dependent on alkyl chain length both for uptake and for concentration at the cellular target.

In addition to total inhibition achieved in studies shown in Table 9.2, others have reported variable results with partial or no inhibition. For example, Martin et al. (1972) found no inhibition of *Alcaligenes viscolactis* in skim milk with up to 600 µg/ml propyl paraben. Moustafa and Collins (1969) found that, whereas 4000 µg/ml propyl paraben inhibited the growth of *Pseudomonas fragi*, 2000 µg/ml actually stimulated growth. Klindworth et al. (1979) demonstrated that 500 µg/ml of a 3:1 mixture of methyl and propyl paraben inhibited vegetative growth of the Gram-positive spore former *Clostridium perfringens*. At 200 µg/ml, propyl paraben inhibited protease secretion by *Aeromonas hydrophila* (Venugopal et al., 1984). Ahmady et al. (1999) found no difference in susceptibility of pathogenic and nonpathogenic strains of *Yersinia* to methyl paraben despite the fact that the same strains showed differences in resistance to certain antibiotics and cationic biocides.

Darwish and Bloomfield (1997) evaluated the effect of cosolvents ethanol, propylene glycol, and glycerol on the activity of methyl and propyl parabens against *Staphylococcus aureus* and

TABLE 9.2
Concentration Ranges of Esters of p-Hydroxybenzoic Acid Necessary for Total Inhibition of Growth of Various Bacteria (pH, Incubation Temperature, and Time Vary)

Microorganism	Concentration ($\mu\text{g/ml}$)				
	Methyl	Ethyl	Propyl	Butyl	Heptyl
Gram-Positive					
<i>Bacillus cereus</i>	1000–2000	830–1000	125–400	63–400	12
<i>Bacillus megaterium</i>	1000	—	320	100	—
<i>Bacillus subtilis</i>	1980–2130	1000–1330	250–450	63–115	—
<i>Clostridium botulinum</i>	1000–1200	800–1000	200–400	200	—
<i>Lactococcus lactis</i>	—	—	400	—	12
<i>Listeria monocytogenes</i>	1430–1600	—	512	—	—
<i>Micrococcus</i> sp.	—	60–110	10–100	—	—
<i>Sarcina lutea</i>	4000	1000	400–500	125	12
<i>Staphylococcus aureus</i>	1670–4000	1000–2500	350–540	120–200	12
<i>Streptococcus faecalis</i>	—	130	40	—	—
Gram-Negative					
<i>Aeromonas hydrophila</i>	550	—	100	—	—
<i>Enterobacter aerogenes</i>	2000	1000	1000	4000	—
<i>Escherichia coli</i>	1200–2000	1000–2000	400–1000	1000	—
<i>Klebsiella pneumoniae</i>	1000	500	250	125	—
<i>Pseudomonas aeruginosa</i>	4000	1000–4000	8000	8000	—
<i>Pseudomonas fluorescens</i>	1310	—	670	—	—
<i>Pseudomonas fragi</i>	—	—	4000	—	—
<i>Pseudomonas putida</i>	450	—	—	—	—
<i>Pseudomonas stutzeri</i>	500–750	400–500	250–300	100	—
<i>Salmonella</i>	2000	1000	1000	1000	—
<i>Salmonella</i> Typhimurium	—	—	180–>300	—	—
<i>Vibrio parahaemolyticus</i>	—	—	50–100	—	—
<i>Yersinia enterocolitica</i>	350	—	—	—	—

Sources: Aalto et al. (1953), Bargiota et al. (1987), Dymicky and Huhtanen (1979), Eklund et al. (1981), Eklund (1985a), Jurd et al. (1971), Juneja and Davidson (1993), Kato and Shibasaki (1975), Lee (1973), Lewis and Jurd (1972), Lück and Jager (1997), Moir and Eyles (1992), Moustafa and Collins (1969), Payne et al. (1989), Pierson et al. (1980), Reddy et al. (1982), Robach and Pierson (1978), Sokol (1952), Tattawasart et al. (1999), Venugopal et al. (1984).

Pseudomonas aeruginosa. It is interesting that the antimicrobial activity of the parabens increased with increasing hydrophobicity of the cosolvent, being greatest with the most hydrophobic cosolvent, ethanol. In contrast, the uptake of parabens was apparently determined by the hydrophilicity of the cosolvent, with glycerol causing the greatest uptake by the cells. Uptake, however, was not correlated with inhibition. It was concluded that inhibition was a combination of the action of the solvent and the paraben on the integrity of the outer (*P. aeruginosa*) and cytoplasmic (both genera) membranes.

Moir and Eyles (1992) compared the effectiveness of methyl paraben and potassium sorbate on the growth of four psychrotrophic foodborne bacteria: *A. hydrophila*, *Listeria monocytogenes*, *Pseudomonas putida*, and *Yersinia enterocolitica*. At pH 5, little difference was found between MICs of methyl paraben and potassium sorbate at 5°C or 30°C. At pH 6 however, methyl paraben was effective at a lower concentration than potassium sorbate for all pathogens except *A. hydrophila*, where the two were equal. Little or no adaptation was found to occur when cells were exposed to

subinhibitory concentrations of antimicrobials. At 5°C in the presence of 1000 µg/ml methyl paraben, *A. hydrophila* survived for 1 to 2 days, *P. putida* and *Y. enterocolitica* for 1 to 2 weeks, and *L. monocytogenes* for more than 4 months. Injury occurred with *A. hydrophila* and *L. monocytogenes* but was variable for *Y. enterocolitica* and did not occur for *P. putida*. Razavilar and Genigeorgis (1998) studied the influence of temperature, time, and inoculum on the efficacy of methyl paraben against growth of *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, and *L. seeligeri*. Methyl paraben at 0.1% allowed growth of all species of *Listeria* at pH 6.0 to 6.2 in Brain Heart Infusion (BHI) broth at 20°C and 30°C. In contrast, no growth occurred with any species at 4°C or 8°C and 0.1% methyl paraben. At 0.15% and 20°C, methyl paraben had inhibitory effects (e.g., increased lag time, decreased final growth level) on the *Listeria* species but did not completely inhibit growth of any species except *L. ivanovii*. Fyfe et al. (1998) evaluated the antimicrobial activity of 0.1% methyl paraben or benzoic acid with plant oil extracts (fennel, anise, or basil) against *Listeria monocytogenes* and *Salmonella* Enteritidis. Methyl paraben, by itself, did not inhibit the growth of either microorganism under the test conditions. However, when combined with 0.2% anise, fennel, or basil oil, *L. monocytogenes* was reduced by 5.1, 5.7, and >8 logs compared to the control after 24 hours, respectively. The combinations were even more effective against *Salmonella* Enteritidis, reducing viable cells by >8 logs compared to the control with all combinations at 24 and 48 hours. All combinations containing methyl paraben were more inhibitory than those containing benzoic acid. This was not surprising because the tests were done in a microbiological medium with a pH around 7.0.

Propyl paraben was tested against *Listeria monocytogenes* Scott A in chicken and hot dog suspensions (Dje et al., 1989). With 10% meat suspensions, propyl paraben effectiveness was dependent on the product. In chicken, *L. monocytogenes* was inhibited 99.9% compared to the control after 24 hour incubation at 35°C. In contrast, little inhibition of *L. monocytogenes* growth was demonstrated by propyl paraben in the hot dog suspension. The difference in effectiveness was theorized to be the result of a higher fat content in the hot dogs. Dje et al. (1990) evaluated the effect of 0.1% propyl paraben and 0.1% methyl:propyl paraben in salt brine (13%) on *L. monocytogenes* suspended in the brine or inoculated on hot dogs that were dipped in brine. Propyl paraben alone had little or no effect on *L. monocytogenes* survival in salt brine at 4°C or on the surface of hot dogs that were dipped in brine for 5 minutes and incubated at 24°C. In contrast, 0.1% methyl:propyl paraben caused a 2 to 3 log decrease in viable *L. monocytogenes* in the salt brine at 4°C. On hot dogs, the antimicrobial combination was less effective, delaying growth for around 4 hours at 24°C of one of two strains tested. In a similar study by Blom et al. (1997), propyl paraben added to vacuum-packaged sliced ham or sausage inoculated with *Listeria monocytogenes* and stored at 4°C or 9°C for 5 weeks was ineffective in controlling the microorganism.

Robach and Pierson (1978) investigated the effect of methyl and propyl paraben on toxin production of *Clostridium botulinum* NCTC 2021. At 100 µg/ml of methyl and 100 µg/ml of propyl paraben, toxin formation was prevented, whereas 1200 µg/ml methyl and 200 µg/ml propyl were necessary for growth inhibition. Reddy and Pierson (1982) and Reddy et al. (1982) determined the effect of methyl, ethyl, propyl, and butyl parabens on growth and toxin production of ten *Clostridium botulinum* strains (5 Type A, 5 Type B). In microbiological medium at pH 7.0 and 37°C, 1000 µg/ml methyl paraben blocked growth and toxin formation for only 1 day. Ethyl and propyl paraben, at the same concentration, prevented growth and toxin production for the maximum incubation time of 7 d. Butyl paraben, as might be expected, was most effective and prevented growth and toxin production for 7 d at 200 µg/ml. Reddy and Pierson (1982) also evaluated ethyl, propyl, and butyl parabens in microbiological medium in the presence of 0.05 M phosphate buffer at pH 7.0 and 6.0. Ethyl paraben prevented growth and toxin production by *C. botulinum* at 37°C for 7 days at 1000 µg/ml (pH 7.0) and 800 µg/ml (pH 6.0). Propyl paraben at 800 µg/ml and 400 µg/ml, and butyl paraben at 200 µg/ml and 100 µg/ml, were similarly effective against *C. botulinum* at pH 7.0 and 6.0, respectively. Draughon et al. (1982) demonstrated effective growth inhibition of *C. botulinum* by 1000 µg/ml of all esters of the parabens. Ethyl paraben at 1000 µg/ml was also effective in

TABLE 9.3
Concentration Ranges of Esters of p-Hydroxybenzoic Acid Necessary for Total Inhibition of Various Fungi (pH, Incubation Temperature, and Time Vary)

Fungi	Concentration (vg/ml)				
	Methyl	Ethyl	Propyl	Butyl	Heptyl
<i>Alternaria</i> sp.	—	—	100	—	50–100
<i>Aspergillus flavus</i>	—	—	200	—	—
<i>Aspergillus niger</i>	1000	400–500	200–250	125–200	—
<i>Byssochlamys fulva</i>	—	—	200	—	—
<i>Candida albicans</i>	1000	500–1000	125–250	125	—
<i>Debaryomyces hansenii</i>	—	400	—	—	—
<i>Penicillium digitatum</i>	500	250	63	<32	—
<i>Penicillium chrysogenum</i>	500	250	125–200	63	—
<i>Rhizopus nigricans</i>	500	250	125	63	—
<i>Saccharomyces bayanus</i>	930	—	220	—	—
<i>Saccharomyces cerevisiae</i>	1000	500	125–200	32–200	25–100
<i>Torula utilis</i>	—	—	200	—	25
<i>Torulasporea delbrueckii</i>	—	700	—	—	—
<i>Zygosaccharomyces bailii</i>	—	900	—	—	—
<i>Zygosaccharomyces bisporus</i>	—	400	—	—	—
<i>Zygosaccharomyces rouxii</i>	—	700	—	—	—

Sources: Aalto et al. (1953), Jermini and Schmidt-Lorenz (1987), Jurd et al. (1971), Kato and Shibasaki (1975), Lewis and Jurd (1972), Marwan and Nagel (1986).

inhibiting toxin formation in canned comminuted pork. However, inhibition of *C. botulinum* by the parabens in many food systems has been reported to be much less than in laboratory media (Sofos and Busta, 1980).

Little research on the activity of the n-heptyl ester in foods has been published. Chan et al. (1975) did show that this compound was very effective in inhibiting bacteria involved in the malolactic fermentation of wines.

FUNGI

The antifungal effectiveness of the parabens has been evaluated against several food-related fungi (Table 9.3). In comparison to bacteria, fungi are much more susceptible to parabens. As with bacteria, inhibition of fungi increases as the alkyl chain length of the parabens increases.

Thompson (1994) evaluated butyl, propyl, ethyl, and methyl parabens, alone and in combination, against strains of mycotoxigenic *Aspergillus*, *Penicillium*, and *Fusarium*. The most effective parabens were the propyl and butyl esters with MICs of 1.0 to 2.0 mM in potato dextrose agar. Combinations of the various parabens were reported to have synergistic activity against the mold species. Nesci et al. (2003) determined that 180 µg/ml propyl paraben partially inhibited conidial germination of *Aspergillus flavus* at a_w of 0.982 and totally inhibited aflatoxin B₁ production. Torres et al. (2003) tested propyl paraben as a potential inhibitor of growth and toxin production by *Fusarium* species on maize. Propyl paraben at 500 µg/ml significantly increased the lag phase and decreased the growth rate of *F. verticillioides* and *F. proliferatum* at a_w of 0.95, 0.98, and 0.995. In addition, 500 µg/ml propyl paraben was effective in totally inhibiting production of fumonisin toxin production by both species at 0.95 a_w . At a_w of 0.98 and 0.995, fumonisin production was reduced 94% to 98% and 20% to 30%, respectively, by 500 µg/ml propyl paraben.

Jermini and Schmidt-Lorenz (1987) evaluated ethyl paraben against osmotolerant yeasts at various water activities and pH levels. They found that the concentration of ethyl paraben necessary for inhibition was a function of initial number of yeast cells present. At 600 $\mu\text{g/ml}$ ethyl paraben, the time to initiate growth was approximately 15, 12, 5, and 2 to 3 days for 10^2 , 10^3 , 10^4 , and 10^5 cells at a_w 0.900 and pH 4.8. Inhibition by parabens was also a function of pH, with more acid environments requiring less ethyl paraben for inhibition. Little effect of water activity or type of humectant was observed. Of the genera tested, *Zygosaccharomyces bailii* was most resistant requiring 900 $\mu\text{g/ml}$ at 25°C, a_w 0.900, and pH 4.8. Other yeasts evaluated included *Torulasporea delbrueckii*, *Z. rouxii*, *Z. bisporus*, and *Debaryomyces hansenii* with MICs of 700 $\mu\text{g/ml}$, 700 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, and 400 $\mu\text{g/ml}$, respectively. They concluded that the concentration of ethyl paraben required to preserve a product from the effect of osmotolerant yeast for 30 days at 25°C and a_w 0.795 to 0.980 was 900 $\mu\text{g/ml}$ or 400 $\mu\text{g/ml}$ at a pH of 4.8 or ≤ 4.0 , respectively.

COMPARISONS TO OTHER PRESERVATIVES

Because parabens are much less influenced by pH, they would seem to be of greater overall value than other "pH-sensitive" antimicrobials in foods, especially those with a near neutral pH. Eklund (1985a) showed that, although antimicrobial activity of parabens was related to pH, this dependence was not related to dissociation of the compounds. Propyl paraben has been shown to be two to eight times more effective at inhibiting bacterial growth than sodium benzoate or sorbate at pH 6.8 to 7.0 (Aalto et al., 1953; Jurd et al., 1971).

MECHANISM OF ACTION

Although an exact cause-and-effect relation for the mode of action of parabens has not been established, various studies have shown that these compounds may be most active at the cytoplasmic membrane. Leakage of intracellular compounds could indicate disruption of the cytoplasmic membrane. Furr and Russell (1972) detected leakage of intracellular RNA (ribonucleic acid) by *Serratia marcescens* in the presence of the parabens. The amount of leakage was proportional to the alkyl chain length of the paraben. Freese et al. (1973) found that the parabens inhibited serine uptake as well as the oxidation of α -glycerol phosphate and NADH (nicotinamide adenine dinucleotide) in membrane vesicles of *Bacillus subtilis*. They concluded that the parabens were capable of inhibiting both membrane transport and the electron transport system. Eklund (1980) did a similar study using *E. coli*, *B. subtilis*, and *P. aeruginosa*. He determined the uptake of alanine by whole cells, and he determined alanine, serine, phenylalanine, and glucose by vesicles. Parabens generally caused a decrease in amino acid uptake but not in glucose uptake. Eklund (1980) postulated that because the parabens are known to cause leakage of cellular contents, they are capable of neutralizing chemical and electrical forces that establish a normal membrane gradient. In continued work with *E. coli*, Eklund (1985b) found that parabens eliminated the ΔpH of the cytoplasmic membrane of the organism. In contrast, the compounds did not significantly effect the $\Delta\Psi$ (membrane potential) component of the proton motive force. He concluded that neutralization of the proton motive force and subsequent transport inhibition could not be the only mechanism of inhibition for the parabens.

Oka (1960) suggested that parabens act on yeast by absorbing on the solid phase of cells rather than in the cell fluid or lipid layers. This conclusion was reached although there was a direct relation between the antimicrobial dissolved in the lipid phase and the minimum concentration necessary to inhibit the yeast. Bargiota et al. (1987) examined the relationship between lipid composition of *S. aureus* and resistance to parabens. Differences were found for total lipid, phospholipids, and fatty acids between *S. aureus* strains, which were relatively resistant and sensitive to parabens. The paraben-resistant strain was shown to have a higher percentage total lipid, higher relative percentage of phosphatidyl glycerol, and decreased cyclopropane fatty acids than sensitive strains. It was

suggested that these changes could influence membrane fluidity and therefore adsorption of the parabens to the membrane. Juneja and Davidson (1993) altered the lipid composition of *L. monocytogenes* by growth in the presence of added fatty acids (C14:0, C18:0, or C18:1). Growth of *L. monocytogenes* in the presence of exogenously added C14:0 or C18:0 fatty acids increased the resistance of the cells to tertiary butyl hydroquinone (TBHQ) and parabens. However, growth in the presence of C18:1 led to increased sensitivity to the antimicrobial agents. Results indicated that, for *L. monocytogenes*, a correlation existed between lipid composition of the cell membrane and susceptibility to antimicrobial compounds.

Rico-Munoz et al. (1987) investigated the effect of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), TBHQ, propyl gallate, p-coumaric acid, ferulic acid, caffeic acid, and methyl and propyl paraben on the membrane-bound ATPase (adenosine triphosphate) of two strains of *S. aureus*. Only BHA was found to significantly stimulate the activity of the enzyme. Propyl gallate, TBHQ, p-coumaric, and ferulic and caffeic acids caused inhibition of ATPase activity. There was no effect on the enzyme with BHT or the parabens. The authors concluded that phenolic compounds probably do not have the same mechanism of action and there may be several targets that lead to inhibition of microorganisms by these compounds.

Resistance of bacteria to the parabens was studied by Valkova et al. (2002). They reported that a strain of *Enterobacter cloacae* produced an esterase that was responsible for the hydrolysis of parabens. The chromosomal gene *prbA* encoded for the production of the esterase, PrbA (Valkova et al., 2003). Valkova et al. (2002) further demonstrated that 41 strains of *Enterobacter gergoviae*, *E. aerogenes*, *E. coli*, *Pseudomonas agglomerans*, *P. aeruginosa*, and *Burkholderia cepacia* also were resistant to methyl paraben but that only two strains of *E. gergoviae* degraded methyl paraben using a similar mechanism. Although these microorganisms were isolated primarily from cosmetics and mineral supplements and as clinical isolates, they illustrate the potential for development of resistance by target foodborne microorganisms.

REGULATORY STATUS

In the United States, the methyl (21 CFR 184.1490) and propyl (21 CFR 184.1670) esters of p-hydroxybenzoic acid are generally recognized as safe (GRAS) at a maximum concentration of 0.1% each (Code of Federal Regulations, 2003). When used in combination, the total may not exceed 0.1%. Both methyl and propyl paraben are permitted as antimycotic agents in food-packaging material (21 CFR 181.23). The n-heptyl (21 CFR 172.145) ester is also approved for use in fermented malt beverages (beers) at a maximum of 12 µg/ml and in certain noncarbonated soft drinks and fruit-based beverages at a maximum of 20 µg/ml. In the European Union, the methyl (E 218), ethyl (E 214), and propyl (E 216) esters are permitted for use in foods. Many other countries permit the use of the methyl and propyl esters, and some, including Japan, allow use of the butyl ester. The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (2000) lists specifications for the methyl (INS 218), butyl, ethyl (INS 214), and propyl (INS 216) esters of the parabens.

APPLICATIONS

To take advantage of their respective solubility and increased activity, methyl and propyl parabens are normally used in a combination of 2:1 to 3:1 (methyl:propyl). Chichester and Tanner (1972) recommended initial testing in foods with 0.05% of a 2:1 methyl:propyl paraben combination. In high-fat foods, they recommended a 0.1% methyl:propyl combination.

The compounds may be incorporated into foods by dissolving in water, ethanol, propylene glycol, or the food product itself. To make an aqueous solution, the water may be at room temperature; however, hot water (70°C to 82°C) is recommended (Chichester and Tanner, 1972).

TABLE 9.4
Applications of the Parabens in Food Products

Product(s)	Ester(s)	Concentration
Bakery products ^a (cakes, crusts, pastries, toppings, fillings)	Methyl:propyl (3:1)	0.03% to 0.06%
Beverages (soft drinks)	Methyl:propyl (2:1)	0.03% to 0.05%
Beer	n-Heptyl; butyl	12 ppm; 0.01%
Fish (marinated, smoked, jellied)	Methyl, propyl	0.03% to 0.06%
Flavor extracts	Methyl, propyl	0.05% to 0.1%
Fruit products	Methyl:propyl (2:1) + sodium benzoate	0.05%
Gelatin	Methyl (or combination)	0.05% to 0.1%
Jams, jellies, preserves (artificially sweetened)	Methyl:propyl (2:1)	0.07%
Malt extracts	Methyl:propyl (2:1) Propyl	0.05% 0.04%
Olives	Methyl, propyl + sodium benzoate	0.1%
Pickles	Methyl:propyl (2:1)	0.1%
Salad dressings	Methyl:propyl (2:1)	0.1%
Sorbitol	Methyl:propyl (2:1)	0.07%
Syrups	Methyl Propyl	0.07% 0.02%
Tomato concentrate	Methyl (with sulfur dioxide and benzoate)	100 µg/ml
Wines	Methyl:propyl (2:1)	0.1%

^a Not for use in yeast-leavened products.

Sources: Chichester and Tanner (1972), Dan et al. (1996), Sethi (1994).

The compounds may also be dry blended with other water-soluble components before addition to the food product. Parabens may be dissolved in ethanol or propylene glycol to make a 10% to 20% stock solution.

Parabens have been suggested for use in a variety of foods (Table 9.4). However, the compounds do not seem to be used widely in foods. According to Lück and Jager (1997), parabens have a perceptible taste at use concentrations; however, other sources refute this (Aalto et al., 1953; Mallinckrodt, n.d.). In addition to products in Table 9.4, other products reported to have been tested with parabens include margarine, butter, ices, confections, soy sauce, maple syrup, and meats (Chichester and Tanner, 1972).

With the development of antimicrobial packaging, the parabens have been studied for their potential incorporation in polymeric films. Dobias et al. (2000) incorporated ethyl and propyl parabens into a low-density polyethylene film at 5 and 10 mg/kg. The parabens were found to migrate into a model water and olive oil food system. Chung et al. (2001a,b) studied the release and inhibitory activity of propyl paraben from a styrene-acrylate copolymer coating. They demonstrated that the propyl paraben not only released from the polymer coating but was capable of inhibiting the growth of *Saccharomyces cerevisiae* better than direct addition of the compound.

TOXICOLOGY

Detailed reviews on the toxicologic aspects of methyl and propyl paraben were published by Soni et al. (2001, 2002).

The acute toxicity of the parabens has been determined to be of a low order. Matthews et al. (1956) found the oral LD₅₀ values in mice for methyl and propyl paraben to be >8000 mg/kg body weight. The sodium salts of methyl, ethyl, propyl, and butyl paraben elicited oral LD₅₀ values of 2000, 2500, 3700, and 950 mg/kg, respectively. The chief toxic effect in dogs was acute myocardial depression with hypotension, but the effects were transient and noncumulative.

In subchronic testing, 500 mg/kg of methyl paraben caused no ill effects to rabbits over a 6-day period, whereas 3000 mg was toxic to the animals (Lück and Jager, 1997). Lück and Jager (1997) also reported that propyl paraben showed similar subchronic toxicity responses. Prindle (1983) reported that feeding 2 to 20 mg/kg per day of the "lower" esters of parabens to rabbits, guinea pigs, or rats caused no harmful effects after 120 days. Rats fed up to 60 mg/kg per day for 30 days also showed no effects.

For chronic toxicity testing, white rats were fed diets containing 2% (0.9 to 1.2 g/kg/day) and 8% (5.5 to 5.9 g/kg/day) each of methyl and propyl paraben (Matthews et al., 1956). After 96 weeks, the animals at the 2% level had no depressed weight gains or any histologic changes in internal organs. At 8% of the diet, however, a mild growth retardation was observed. The same researchers found that mongrel dogs could tolerate daily doses of 1 g/kg of the methyl and propyl esters for 1 year with no ill effects. Tissue samples from these animals were normal.

The parabens are absorbed from the gastrointestinal tract, and the ester linkage is hydrolyzed in the liver and kidney (Jones et al., 1956). The resulting p-hydroxybenzoic acid is excreted in the urine unchanged or as p-hydroxyhippuric acid, glucuronic acid esters, or sulfates (Lück and Jager, 1997). A majority of the metabolites of the parabens are excreted within 6 and 24 hours following intravenous and oral doses, respectively (Jones et al., 1956).

Matthews et al. (1956) reported that none of the esters produced skin irritation in humans at concentrations of 5%. Epstein (1968), however, reported that parabens in foods were associated with a dermatitis. Contact dermatitis involving parabens has been reported, although it is associated with topical use (Reitschel and Fowler, 2001). The concentrations required to elicit such a response are generally high, and no mechanism is known for the sensitivity (Soni et al., 2001). Similarly, allergic reactions have been reported with parabens, but evidence of allergenicity of the compounds is lacking (Soni et al., 2001).

ASSAY

Several methods are available for the qualitative or quantitative determination of the parabens. Chichester and Tanner (1972) describe a qualitative thin-layer chromatographic technique using kieselguhr-silica gel plates and a hexane-acetic acid solvent system. The compounds are first separated from an acidified food system using steam distillation followed by solvent extraction. After development, the plates are observed under ultraviolet light to detect the parabens.

Another technique described by Lück and Jager (1997) involves extraction of a food with ether-petroleum ether mixtures or an acidified steam distillation system. The esters are then saponified and determined spectrophotometrically as p-hydroxybenzoic acid at 255 nm.

According to the Joint FAO/WHO Expert Committee on Food Additives (2000), 2 g of a dried sample to be assayed for parabens should be weighed to the nearest milligram and transferred to a flask. Forty milliliters of 1 N sodium hydroxide are added, and the sides of the flask are rinsed with water. The flask is covered with a watch glass, and the solution is boiled gently for 1 hour and cooled. Five drops of bromothymol blue are added, and the mixture is titrated 1 N sulfuric acid, comparing the color with a buffer solution (pH 6.5) containing the same proportion of indicator. Blank determinations with the reagents are performed to make any necessary corrections. Each milliliter of 1 N sodium hydroxide is equivalent to 152.2 mg of (methyl) C₈H₈O₃, 166.18 mg of (ethyl) C₉H₁₀O₃, or 180.2 mg of (propyl) C₁₀H₁₂O₃.

Lin and Choong (1999) devised a method for simultaneous determination of seven preservatives including methyl, ethyl, propyl, and butyl parabens along with benzoic acid, sorbic acid, and dehydroacetic acid in vinegar, soy sauce, pickle condiment, and fish sauce. The method used is a direct injection gas chromatographic technique with an intermediate polar column. The method was successful in recovering $\geq 95\%$ of the compounds spiked into samples.

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10 Dimethyl Dicarbonate and Diethyl Dicarbonate

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CONTENTS

Regulatory Status	306
Chemistry	307
Description	307
Ethyl Carbamate Formation.....	307
Synthesis	308
Reactions.....	308
Antimicrobial Activity	309
Yeast.....	309
Bacteria	310
Molds	310
Physical and Chemical Effects on Activity.....	310
Temperature.....	310
pH.....	312
Ethanol and Other Constituents.....	312
Mechanism of Action.....	313
Applications	314
Fruit Juices.....	316
Grape Juice and Wines	317
Soft Drinks.....	318
Foods.....	318
Beer	319
Toxicology.....	319
Foods.....	319
Direct Exposure	320
Indirect Exposure.....	320
Analysis.....	320
Direct.....	320
Indirect	321
References	322

Diethyl dicarbonate (DEDC), commonly called diethyl pyrocarbonate, has been mentioned in the Russian literature as a cause for effervescence in sparkling wine (Parfentjev and Kovalenko, 1951a,b; Merzhanian, 1951). Kozenko (1952), in further discussing DEDC, mentioned that in 1933 a Russian investigator expressed the opinion that the neutral esters of carbonic acid were involved

in sparkling wine effervescence. Although these works were erroneous in their conclusions, they stimulated interest in these types of compounds.

Boehm and Mehta (1938) first isolated and properly identified the alkyl esters of pyrocarbonic acid. Bayer AG first introduced DEDC into the market for trial tests about 1959, at which time they also applied for a U.S. patent (Bernhard et al., 1959). Hennig (1959) reported on the effectiveness of DEDC as a fungicide in wine and also reported that it caused no off-aromas or off-flavors. Before the introduction into the market, Bayer AG tested a number of analogs of DEDC. The choice of DEDC over the equally effective fungicide dimethyl dicarbonate (DMDC) was made, according to Genth (1972), because of the innocuous hydrolysis products, ethanol and carbon dioxide. However, a 1972 ban on the use of DEDC in the United States resulted in greater focus on DMDC.

REGULATORY STATUS

DEDC may not be used for treatments involving food or beverages in the United States. In 1972, based on a report by Löfroth and Gejvall (1971), the U.S. Food and Drug Administration (FDA) banned the use of DEDC (FDA, 1972). Until that time, it was legally used in a number of beverages. The first approved use for DMDC was for addition to wines as a yeast inhibitor at a level not to exceed 200 ppm (FDA, 1988). The FDA, in 2001, amended the food additive regulations to provide for a more descriptive term, "microbial control agent," in place of "inhibitor of yeast," for the safe use of DMDC based on a food additive petition filed by Bayer Corp. (FDA, 2001). DMDC presently is approved for use as a direct food additive to be used as a microbial control agent in certain beverages in which the microbial population has been reduced to 500 microorganisms per mL or less by current good manufacturing practices. Beverages in which DMDC is approved for use are as follows (FDA, 2001):

1. Wine, dealcoholized wine, and low alcohol wine, at a limit of 200 ppm
2. Ready-to-drink teas, at a limit of 250 ppm
3. Carbonated or noncarbonated, nonjuice-containing ($\leq 1\%$ juice), flavored or unflavored beverages containing added electrolytes (5 to 20 mEq/L Na^+ and 3 to 7 mEq/L K^+), at a limit of 250 ppm
4. Carbonated, dilute beverages containing juice, fruit flavor, or both, in which the juice content does not exceed 50%, at a limit of 250 ppm

In 2000, Bayer Corp. filed an effective notification of food contact substance (FCN 0035) with the FDA, thereby allowing for use of DMDC as a microbial control agent in noncarbonated juice beverages containing up to and including 100% juice (FDA, 2000). A food contact substance is defined as one intended for use as a component of material used in manufacturing, packaging, transporting, or holding food where the substance is not intended to have a technical effect on the food (FDA, 2002). Limitations for use of DMDC as a food contact substance are similar to those specified for its use as a food additive (i.e., the microbial load of the juice must be reduced by current technologies [e.g., heat] prior to addition of DMDC at a limit of 250 ppm) (FDA, 2003). DMDC is marketed and sold for application to foods under the registered trademark Velcorin®.

The basis for the ban on DEDC was that a small amount of ethyl carbamate, a carcinogen, was formed by the reaction of ammonia with the DEDC. Despite extensive proof of the error of that report (Fischer, 1972; Ough, 1976a), the ban was upheld, based on the Delaney clause of the Food Additive Amendment. It is interesting that *all* fermented products tested in one study, including yogurt, beer, and soy sauce, had small but detectable amounts of ethyl carbamate (Ough, 1976a). As it turns out, the amount of ethyl carbamate added to the food chain by the use of DEDC would be minor. The natural formation of precursors in fermented foods and beverages and in drugs using

alcohol as either a carrier or as an antiseptic far exceeds any formed from the use of DEDC. Nevertheless, this has not altered the FDA's position on this compound.

CHEMISTRY

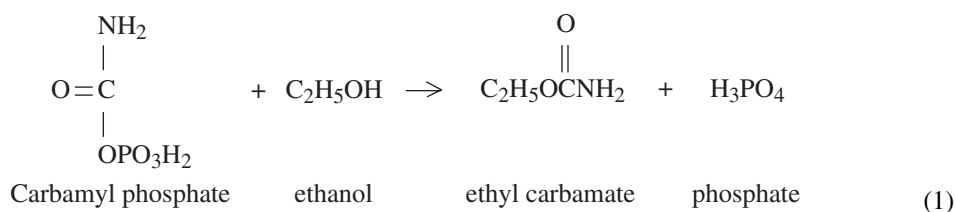
DESCRIPTION

DEDC is a colorless liquid with a faint fruitlike odor. The boiling point at 760 mm Hg is 155°C, at which temperature it decomposes. The density of DEDC is 1.12 g/cm³ at 20°C, the refractive index is 1.397 at 25°C, and the viscosity is 1.97 cP at 20°C. It is only slightly soluble in water, with a solubility of about 0.6 g per 100 g at 18°C. The compound also hydrolyzes rapidly in water. In ethanol, solubility is greater (50 g per 100 g) and hydrolysis is slower. DEDC is a lacrimator and causes skin irritation.

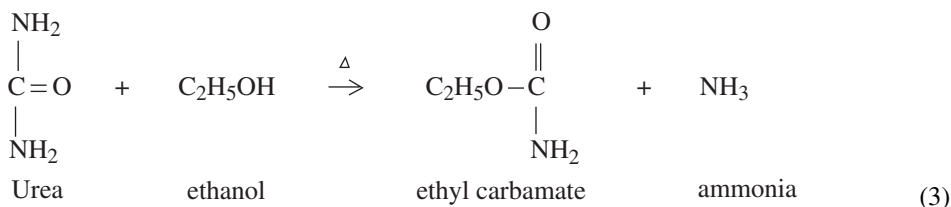
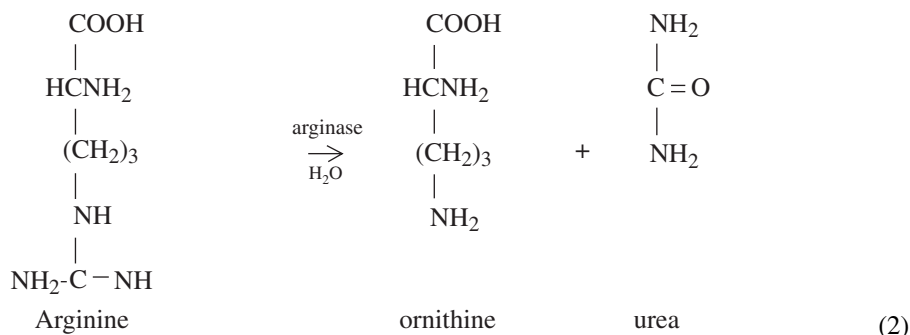
DMDC is a colorless, fruity-smelling liquid, and it has a melting point of 15.2°C, a boiling point of 123°C to 149°C, with decomposition, and a density of 1.26. It is also only slightly soluble in water and more soluble in organic solvents. Skin and eye contact should be avoided.

ETHYL CARBAMATE FORMATION

One source of this compound is the reaction of carbamoyl phosphate with ethanol. This ubiquitous compound, of the ornithine cycle, is found in all living cells. The reaction is as follows:



It has been conclusively shown by Ough et al. (1988b, 1990) and Monteiro et al. (1989) that fermenting yeasts metabolize arginine and excrete urea into the medium. This is the primary source in wine.



SYNTHESIS

The synthesis of dialkyl dicarbonate esters was described by Boehm and Mehta (1938). Their method involved the heating of the chlorocarbonic ethyl esters of emetine (6', 7', 10, 11-tetromethoxyemetan), an alkaloid, with dilute potassium hydroxide. DEDC could be formed, as well as some other dialkyl dicarbonates, but DMDC could not be recovered. Kovalenko (1952) devised a more direct method and was able to synthesize DMDC. The reaction used was the following:

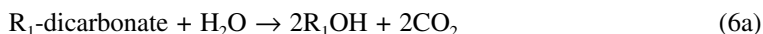


REACTIONS

The diethyl and dimethyl dicarbonates are extremely reactive substances. Some of the reactions that can take place are as follows:



(R = either two methyl groups or two ethyl groups).



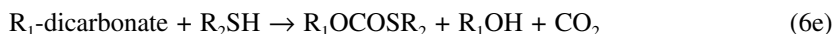
(R₂ = any alkyl or aromatic group).



(R₂ = alkyl group, alkyl-C-, aromatic, or H).



(R₂ = any alkyl or aromatic group).



(R₂ = any alkyl or aromatic group).

The hydrolysis reaction (6a) was recognized by Kovalenko (1952). The alcoholysis (6b) was documented by Kielhöfer and Würdig (1963a). Larrouquère (1963) reacted alkyl and aromatic amines with DEDC to produce reaction (6c). Thoukis et al. (1962) reported reactions of DEDC with -NH₂ groups and noted carbethoxy compounds could be formed with amino acids. The mixed esters formed by reaction of DEDC with the desired alkyl acid (6d) were indicated by Thoma and Rinke (1959). Mühlrad et al. (1967) stated that the sulfydryl groups of cysteine were carbethoxylated by DEPC (6e). Larrouquère (1965) also investigated the reaction of DEDC with thiol groups. Similar reactions [(4) through (6)] for DMDC have been reported by Peterson and Ough (1979), Ough and Langbehn (1976), and Stafford and Ough (1976).

Other reactions of DEDC that have been investigated include those with phenols and phenol glucosides (Paulus and Lorke, 1967), those with vitamins (Fischer, 1970), and those with malic acid (Schelenz and Fischer, 1971). The report of Duhm et al. (1966) indicated that a large range of food chemicals reacted with DEDC.

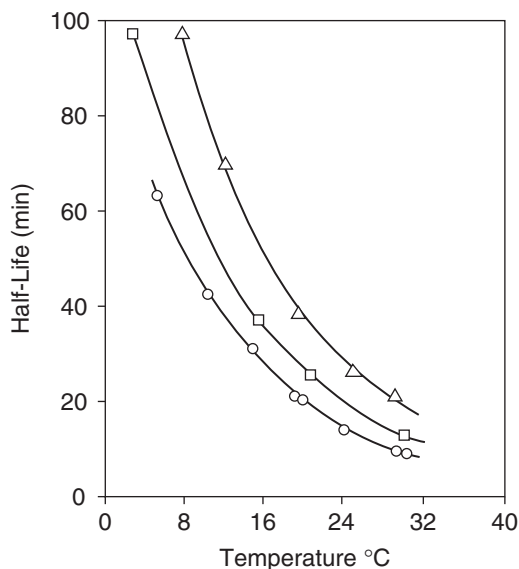


FIGURE 10.1 Hydrolysis of DEDC (diethyl dicarbonate) and DMDC (dimethyl dicarbonate) at different temperatures. Half-life refers to the time for half of the DEDC or DMDC to hydrolyze: DMDC in water (*circles*); DMDC in 14.6% vol/vol ethanol (*squares*); DEDC in water (*triangles*).

The hydrolysis rate of DEDC was considered by Pauli and Genth (1966) and further by Schelenz and Fischer (1970). The kinetics of hydrolysis of DMDC have been reported by Peterson (1978) and Genth (1979). Figure 10.1 gives the half-life of DMDC and DEDC in water and that of DMDC in 14% vol/vol ethanol at a variety of temperatures.

ANTIMICROBIAL ACTIVITY

After the initial publications of the effectiveness of DEDC against wine yeast, a number of investigators further examined its usefulness as a bactericide and fungicide. Because the dicarbonates rapidly hydrolyze to the corresponding alcohol (methyl or ethyl) and carbon dioxide, the challenge is to destroy target microorganisms quickly, before hydrolysis is completed. As such, the dicarbonates cannot be relied on for long-term protection against recontamination or later outgrowth of surviving organisms.

YEAST

Reviews by Pauli and Genth (1966) and Genth (1964) included numerous reports on the fungicidal action of DEDC on yeasts. Their fungicidal data plus those of Alimukhamedova (1975), Grospicova et al. (1969), and Todor et al. (1967) are shown in Table 10.1. Much of the differences in antifungal effectiveness of DEDC are the result of the conditions under which each test was made. Test conditions as well as tolerance differences between genera, species, or even strains could account for most of the variation.

Studies with DMDC are not as extensive as those for DEDC. Genth (1979) reported the effectiveness of DMDC as a fungicide when used in alcohol-free beverages (Table 10.2). Daudt and Ough (1980) reported its use as a sterilant against yeast in wine (Table 10.3). DMDC has proved to be equal or superior to DEDC as a fungicide in both instances.

Pauli and Genth (1966) showed that, for every 10-fold increase in cell count, the amount of DEDC required for fungicidal action was doubled. Daudt and Ough (1980) showed that 50 mg/L

of DMDC effectively sterilized a wine with 10% vol/vol ethanol containing 2×10^4 cells. Viable cells remained, however, if only 25 mg/L of DMDC were used, with as few as 50 cells/mL in the wine initially.

The rate of kill is proportional to the concentration of DEDC (Ough and Ingraham, 1961). More than 2 hours were required for complete kill of 50 yeast cells/mL with the addition of 40 mg/L of DEDC; less than half an hour was required with 120 mg/L of DEDC. Similar results were found using DMDC (Daudt and Ough, 1980). A delay in the lethal effect at lower DEDC or DMDC concentrations could be the result of the capability of the yeast cells to reproduce for short periods before permanent damage and death of the organism.

Ough et al. (1988c) investigated the interaction of sulfur dioxide and DMDC. They found additive effects between the two inhibitors when testing a very resistant yeast. For a lethal dose of DMDC for this yeast, a much larger than normal amount was required.

BACTERIA

A comparison of the antibacterial effectiveness of DEDC on a number of different genera has been summarized in Table 10.4 (Pauli and Genth, 1966). Similar tests using DMDC (Genth, 1979, 1980) are shown in Table 10.5. Results on the antimicrobial activity of these compounds indicated that they had approximately equivalent bactericidal activity (Genth, 1979, 1980).

Murata (1974) found that 1300 mg/L of DEDC was capable of destroying a *Lactobacillus casei* bacteriophage. Ough et al. (1988c) found *Leuconostoc oenos* relatively insensitive to DMDC at normal wine treatment levels. It had a slight additive effect on the bacteria when used with sulfur dioxide.

MOLDS

For activity against molds, higher levels of DEDC were required than for either yeast or bacteria. Table 10.6 shows the results summarized by Pauli and Genth (1966). Genth (1979) reported that 500 spores per milliliter of *Penicillium glaucum*, *Byssochlamys fulva*, *Botrytis cinerea*, *Mucor racemosus*, and *Fusarium oxysporum* required 200, 100, 100, 500, and 100 mg/L of DMDC, respectively, for effective killing. Vegetative cells and mold conidia are relatively sensitive to both dicarbonates. However, heat-resistant structures such as ascospores of *B. fulva* are quite resistant and thus likely to survive exposure to up to 200 ppm DMDC (Splittstoesser and Wilkinson, 1973; Van der Riet et al., 1989).

PHYSICAL AND CHEMICAL EFFECTS ON ACTIVITY

It has been recognized that DEDC activity could be altered by some of the physical or chemical variables existing during handling of products. Such variables are temperature, pH, chemical makeup of product, and the amount of other reactive substances in the treated material.

TEMPERATURE

The hydrolysis rate of DEDC or DMDC is dependent on temperature — the higher the temperature the more rapid the rate. The half-life values for both DEDC and DMDC are given in Figure 10.1. Despite the more rapid hydrolysis at higher temperatures, Turtura (1966) found that increased temperature enhanced the effectiveness of DEDC as a sterilant. He noted that DEDC was twice as effective at 27°C as at 0°C. Grospicova et al. (1969) also studied the effect of treatment temperature and found a twofold increase in effectiveness from 6°C to 20°C on two *Lactobacillus* species. Shibasaki et al. (1969) noted the temperature coefficient for molds in distilled water and in phosphate buffer was 2.5 and 2.3, respectively, but the temperature coefficient for the microbiocidal activity was 5 and 10 for the same two solutions. Their work with *Penicillium thomii* showed that

TABLE 10.1
Fungicidal Activity of Diethyl Dicarbonate^a

Yeast	Starting Cell Concentration per ml	Diethyl Dicarbonate (DEDC) Concentration (mg/L) Sufficient to Be Fungicidal (100% kill)
<i>Saccharomyces acidifaciens</i>	5.05×10^2	200
<i>S. apiculatus</i>	1.0×10^5	60–90
<i>S. cerevisiae</i>	$5.0 \times 10^2 - 1.0 \times 10^4$	30–80
<i>S. cerevisiae</i>	5.0×10^4	50
<i>S. cerevisiae</i>	1.5×10^2	50–100
<i>S. cerevisiae</i>	5.0×10^2	50
<i>S. cerevisiae</i>	4.0×10^3	500–1000
<i>S. cerevisiae</i>	1.0×10^3	500
<i>S. cerevisiae</i>	3.6×10^6	50
<i>S. cerevisiae</i>	1.4×10^4	12
<i>S. cerevisiae</i> var. <i>ellipsoides</i>	5.0×10^4	100
Burgundy		
<i>S. cerevisiae</i> var. <i>ellipsoides</i>	6.0×10^5	100
Champagne		
<i>S. carlsbergensis</i>	4.0×10^3	250–1000
<i>S. chevalieri</i>	5.6×10^2	30
<i>S. globosus</i>	8.0×10^2	30
<i>S. heterogenicus</i>	5.2×10^2	30
<i>S. ludwigii</i>	5.0×10^4	25
<i>S. oviformis</i>	5.0×10^4	50
<i>S. pastorianus</i>	4.0×10^5	100
<i>S. pastorianus</i>	4.0×10^2	100
<i>S. pastorianus</i>	4.0×10^5	100
<i>S. rouxii</i>	1.0×10^5	100–500
<i>S. uvarum</i>	5.0×10^4	25
<i>S. uvarum</i>	2.0×10^3	200
<i>Schizosaccharomyces pombe</i>	5.0×10^2	400
<i>Brettanomyces bruxellensis</i>	1.0×10^5	250
<i>B. vini</i>	1.0×10^4	25
<i>B. claussenii</i>	5.0×10^2	200
<i>B. species</i>	5.0×10^4	25
<i>B. species</i>	4.6×10^3	100
<i>Pichia alcoholopila</i>	1.0×10^3	100
<i>P. farinosa</i>	4.0×10^5	100
<i>P. farinosa</i>	4.0×10^3	100
<i>P. membranefaciens</i>	5.0×10^2	25
<i>P. membranefaciens</i>	2.0×10^4	14
<i>Torula utilis</i>	1.0×10^5	250
<i>Torulopsis candida</i>	1.0×10^5	100
<i>T. colliculosa</i>	5.6×10^2	30
<i>T. colliculosa</i>	5.0×10^2	200
<i>T. versatilis</i>	5.0×10^2	200
<i>Rhodotorula glutinosa</i>	5.6×10^2	30
<i>R. mucilaginoso</i>	1.0×10^5	200
<i>R. rubra</i>	2.0×10^3	200
<i>R. rubra</i>	5.0×10^3	300
<i>Candida krusei</i>	1.0×10^5	60–200

(continued)

TABLE 10.1 (CONTINUED)
Fungicidal Activity of Diethyl Dicarboxylate^a

Yeast	Starting Cell Concentration per ml	Diethyl Dicarboxylate (DED) Concentration (mg/L) Sufficient to Be Fungicidal (100% kill)
<i>C. krusei</i>	5.0×10^2	200
<i>C. krusei</i>	5.0×10^4	200
<i>C. lipolytica</i>	1.0×10^5	90–500
<i>C. mycoderma</i>	5.0×10^2	200
<i>C. mycoderma</i>	2.5×10^3	100
<i>C. parapsilosis</i>	5.0×10^2	200
<i>C. pseudotropicalis</i>	5.0×10^2	200
<i>C. pulcherima</i>	5.0×10^2	200
<i>Cryptococcus diffluens</i>	5.0×10^4	75
<i>Kloeckera apiculata</i>	1.0×10^4	200
<i>Debaryomyces hansenii</i>	5.0×10^2	200
<i>Hanseniaspora apiculata</i>	1.0×10^4	25
<i>Hansenula anomala</i>	4.0×10^3	250–500
<i>H. anomala</i>	5.0×10^2	200
<i>H. anomala</i>	2.0×10^3	100
<i>Fabospora macedoniensis</i>	4.0×10^2	30
<i>Zygosaccharomyces europaeicus</i>	6.3×10^2	30
<i>Zygosaccharomyces</i> sp.	1.0×10^5	80

^a Reports of various tests done under varied environmental conditions.

at pH 4.0, about a fourfold increase in the antiseptic effectiveness of DEDC was evident as the temperature was increased from 10°C to 20°C, and another 10-fold increase occurred at 20–30°C.

Splittstoesser and Wilkinson (1973) reported a 100-fold increase in effectiveness of DEDC against *S. cerevisiae* and *Lactobacillus plantarum* by increasing the temperature from 20°C to 40°C. Goto et al. (1970) found no temperature effect; however, they used only 250 mg/L of DEDC against high numbers of yeast cells. At the higher temperature (35°C), the rapid growth rate could have overcome the sterilization effects in the rich medium used. They also found no effect of ethanol or sugars on the kill rates (Goto et al., 1970).

pH

Reaction of R-NH groups with DEDC and DMDC (one of the proposed modes of action) is dependent on the degree of ionization of the R-NH group. In the pH range 3.0 to 4.0, this relation is demonstrated by the reaction of DEDC and DMDC with ammonia to form urethanes (Figure 10.2). As the ammonia becomes ionized at the lower pH, the reaction is lessened (Ough, 1976b; Ough and Langbehn, 1976).

ETHANOL AND OTHER CONSTITUENTS

The rate of hydrolysis of DEDC or DMDC can be significantly decreased by increasing the ethanol concentration. Other water-soluble organic solvents also have similar effect. The reactivity of DEDC or DMDC is also such that any proteinaceous material suspended in the solution reacts and decreases the antimicrobial effectiveness of the treatment. Porter and Ough (1982) found that ethanol increased the effectiveness of DMDC and that 20°C was the optimum use temperature.

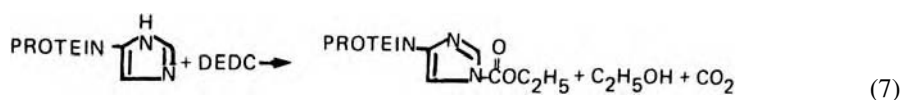
TABLE 10.2
Effective Fungicidal Concentration of DMDC in Alcohol-Free Beverages

Yeast	Dimethyl Dicarbonate (DMDC) Concentration (mg/L) Sufficient to Be Fungicidal (100% kill)
<i>Saccharomyces carlsbergensis</i>	100
<i>S. carlsbergensis</i>	70
<i>S. diastaticus</i>	200
<i>S. oviformis</i>	100
<i>S. bailii</i>	120
<i>S. cerevisiae</i>	30
<i>S. uvarum</i>	20
<i>S. pastorianus</i>	100
<i>S. apiculatus</i>	70
<i>S. globosus</i>	40
<i>Zygosaccharomyces priorianus</i>	90
<i>Rhodotorula mucilaginosa</i>	50
<i>R. glutinosa</i>	30
<i>R. rubra</i>	200
<i>Candida krusei</i>	200
<i>Pichia membranefaciens</i>	30
<i>P. farinosa</i>	100
<i>Torulopsis candida</i>	100
<i>T. versatilis</i>	100
<i>T. stallata</i>	75
<i>Torula utilis</i>	250
<i>Endomyces lactis</i>	70
<i>Kloeckera apiculata</i>	30
<i>Hansenula anomala</i>	50

^a The cell counts were about 500 cells/mL, temperature 28°C, and pH between 2.8 and 4.7.

MECHANISM OF ACTION

The inactivation of microorganisms by DEDC or DMDC seems strongly related to the inactivation of the enzymes of the organism. Protein modification, through reaction of nucleophilic groups, such as imidazoles, amines, or thiols, can readily occur with the dicarbonate (Osterman-Golkar et al., 1974; Ehrenberg et al., 1976). The point of attack is one of the central carbon atoms. The residual portion of the dicarbonate is unstable and decomposes rapidly. Reactions with protein imidazole (7) and amine groups are of particular importance owing to the ease and rapidity with which they occur (Means and Feeney, 1971).



Mühlrad et al. (1967) investigated the reactions of DEDC with a number of amino acids. The reaction with histidine is similar to that of the imidazole reaction (i.e., a reaction with the imino nitrogen). Holbrook and Ingram (1973) found that the enzyme lactate dehydrogenase was completely inactivated by reaction of DEDC with the histidyl groups of the enzyme. Many other reports

TABLE 10.3
Antimicrobial Effectiveness of Dimethyl Dicarboxylate (DMDC) in a Standard Wine Containing 10% Ethanol

Microorganism	DMDC Added (mg/L)					
	0	6.25	12.5	25	50	100
<i>Saccharomyces cerevisiae</i> Montrachet	58 ^a	16	0	0	0	0
<i>S. bayanus</i> Champagne	2500	419	729	0	0	0
<i>Schizosaccharomyces pombe</i>	42	14	4	0	0	0
<i>Saccharomyces cerevisiae</i> Steinberg	96	50	43	0	0	0
<i>S. fermentati</i> Flor ^b	203	53	9	0	0	0
<i>Brettanomyces</i> spp.	10,000	2000	100	0	0	0
<i>Saccharomyces cerevisiae</i> Distillers	78	0	0	0	0	0
<i>S. cerevisiae</i> Geisenheim	88	0	0	0	0	0
<i>S. cerevisiae</i> Burgundy	2000	310	0	0	0	0
<i>S. cerevisiae</i> Rankine 350	89	64	38	0	0	0
<i>S. cerevisiae</i> Eperney Rankine 729	2500	2000	844	0	0	0
<i>S. cerevisiae</i> Tokay ^b	887	129	144	0	0	0
Champagne Epernay (Geisenheim Reinzuchthefen)	5000	3000	350	0	0	0
<i>Saccharomyces bayanus</i> (formerly <i>oviformis</i>)	17	19	0 ^c	0	0	0
<i>S. oviformis</i> (<i>S. bayanus</i>) Inst. Pasteur	214	346	200	11	0	0
<i>Schizosaccharomyces pombe</i> Moscovin ^b	2200	529	2000	4	0	0
Geisenheim 1949 (Geisenheim Reinzuchthefen)	1200	800	640	5	0	0
<i>Dekkera intermedia</i> FS&T 71-12	2000	600	70	0	0	0
<i>Rhodotorula rubra</i>	1000	800	720	650	0	0

^a Values are the number of viable cells remaining in wine after 38 hours.

^b Yeasts did not grow well in wines with 10% ethanol; wines of 8% ethanol were used instead. *S. fermentati* Flor needs to stay at least 4 days in the incubator (at 37°C) to grow on the plates; *S. cerevisiae* Tokay and *S. pombe* Moscovin need 3 days. *S. fermentati* Flor also grew better in 8% ethanol wines, but the data presented are for 10% ethanol wines.

^c Although nothing grew on the plates, growth developed later in the bottles, indicating some viable cells remaining.

(Huc et al., 1971; Dann and Briton, 1974; Choong et al., 1977) with other enzymes have shown that DEDC reacts primarily with the histidyl moiety to form carbethoxy-histidine. Enzyme inhibition results from active site blocking and conformational changes. For example, the histidyl group in alcohol dehydrogenase has been proposed (Ringold, 1966) to be involved in the oxidation-reduction process. Blocking of either nitrogen in the histidine ring would inactivate the enzyme. DEDC has been used routinely to inhibit nuclease in the extradiation of nucleic acids. Any single key enzyme inactivated could eventually cause the death of an organism. A report by Ehrenberg et al. (1976) reviews the many reactions that can take place with DEDC.

APPLICATIONS

Pauli (1984) reviewed the nature of food additives and their possible hazardous reactions. DEDC was one of concern. The usefulness of DMDC as a beverage preservative, its antimicrobial activity, and some of its chemistry were reviewed by Thoukis (1983). Because of its regulatory status, DMDC is approved only for use in various beverages. Furthermore, its application to beverages requires use of special equipment for metering and mixing DMDC.

TABLE 10.4
Antibacterial Effectiveness of Diethyl Dicarbonate (DEDC)^a

Bacteria	Starting Cell Concentration per mL	DEDC Concentration (mg/L) Sufficient to Be Bactericidal (100% Kill)
<i>Acetobacter pasteurianus</i>	5.0×10^2	80
<i>A. xylinum</i>	6.0×10^3	300
<i>Bacterium coli</i>	1.0×10^5	400
<i>Escherichia coli</i>	1.0×10^5	1500
<i>Bacterium aceticum</i>	5.0×10^4	500
<i>Staphylococcus aureus</i>	1.0×10^3	70–100
<i>Lactobacillus arabinosus</i>	1.0×10^6	250
<i>L. helveticus</i>	1.0×10^7	100–300
<i>L. pastorianus</i>	6.0×10^2	300
<i>Lactobacillus buchneri</i>	4.8×10^2	30
<i>Bacillus subtilis</i>	4.0×10^9	3000
<i>Salmonella</i> Typhimurium	1.0×10^5	2000
<i>Bacillus mediosporum</i>	4.8×10^2	30
<i>B. megatherium</i>	4.8×10^2	30
<i>Salmonella</i> Thompson	1.0×10^5	2000
<i>Pseudomonas cocciformis</i>	8.0×10^2	30
<i>Pseudomonas</i> , pigmented	1.0×10^5	200
<i>Pseudomonas</i> , unpigmented	1.0×10^5	500
<i>P. erithrogloem</i>	7.2×10^2	30
<i>P. flavum</i>	4.8×10^2	30
<i>P. mildenbergii</i>	6.4×10^2	30
<i>Micrococcus annulatus</i>	4.8×10^2	30
<i>M. lacteus</i>	6.4×10^2	30
<i>M. lacteus</i>	6.4×10^2	30
<i>Sarcina flavescens</i>	8.0×10^2	30

^a Determined under various environmental conditions.

TABLE 10.5
Antibacterial Effectiveness of Dimethyl Dicarbonate (DMDC)^a

Bacteria	Starting Cell Concentration per mL	DMDC Concentration (mg/L) Sufficient to Be Bactericidal (100% Kill)
<i>Acetobacter pasteurianus</i>	5.0×10^2	80
<i>Escherichia coli</i>	5.0×10^2	400
<i>Pseudomonas aeruginosa</i>	5.0×10^2	100
<i>Staphylococcus aureus</i>	5.0×10^2	100
<i>Lactobacillus buchneri</i>	5.0×10^2	30
<i>Lactobacillus pastorianus</i>	5.0×10^2	300
<i>Lactobacillus brevis</i>	5.0×10^2	200
<i>Pediococcus cerevisiae</i>	5.0×10^2	300

^a Temperature 28°C and pH between 2.8 and 4.7 in an artificial medium.

TABLE 10.6
Fungicidal (Mold) Effectiveness of Diethyl Dicarboxate

Mold	Starting Spore Concentration per mL	DEDIC Concentration (mg/L) Sufficient to Be Fungicidal (100% kill)
<i>Aspergillus flavus</i>	5.0×10^2	1000
<i>A. niger</i>	1.0×10^4	150
<i>A. niger</i>	2.9×10^2	750
<i>A. terreus</i>	3.0×10^2	250–400
<i>Penicillium digitatum</i>	3.0×10^2	120–250
<i>P. expansum</i>	1.0×10^2	100
<i>P. glaucum</i>	3.0×10^2	250
<i>P. italicum</i>	3.0×10^2	100
<i>P. leteum</i>	3.0×10^2	300
<i>Mycoderma species</i>	4.0×10^3	500–1000
<i>Trichoderma viride</i>	1.0×10^3	500
<i>Botrytis cinerea</i>	1.0×10^3	100
<i>Oidium lactis</i>	3.0×10^2	300–700
<i>Fusarium oxysporum</i>	1.0×10^3	100
<i>Fusarium orthoceras</i>	1.0×10^3	100
<i>Neurospora sitophila</i>	3.0×10^2	50–200
<i>Mucor racemosus</i>	3.0×10^2	300–700
<i>Rhizopus nigricans</i>	3.0×10^2	300
<i>Pullularia pullulans</i>	3.0×10^2	100–200
<i>Cladosporium herbarum</i>	5.0×10^2	100
<i>Paecilomyces</i> sp.	1.3×10^2	1000

^a Tested under various environmental conditions.

FRUIT JUICES

Fisher and Golden (1998) reported that *Escherichia coli* O157:H7 populations were reduced from about 7 log CFU/mL to undetectable levels within 3 days in apple cider containing 250 ppm DMDC and stored at 4°C; increasing the storage temperature to 25°C enhanced DMDC effectiveness, and *E. coli* O157:H7 was undetectable within 2 days of storage. Combination of DMDC with sodium benzoate or potassium sorbate increases effectiveness and offers enhanced protection by providing a secondary barrier against surviving spoilage organisms (Golden, 2002; Worobo, 2002). *E. coli* O157:H7 populations were reduced from 7 log CFU/mL to undetectable levels at 4°C within 72 hours in apple cider and 48 hours in orange juice containing 250 ppm DMDC (Lakins, 2002). A combination of 250 ppm DMDC and 450 ppm sodium benzoate rendered *E. coli* O157:H7 undetectable within 48 hours in apple cider and 24 hours in orange juice. Lakins (2002) reported that inoculated *Salmonella* (7 log CFU/mL initial) was undetectable in apple cider containing 250 ppm DMDC within 48 hours; the same result was achieved within 24 hours in cider containing a combination of 250 ppm DMDC and 450 ppm sodium benzoate.

Worobo (2002) reported that strain variation and differences in apple varieties used in cider making affect sensitivity of *E. coli* O157:H7 in apple cider. Using 250 ppm DMDC, *E. coli* O157:H7 D-values ranged from a low of 0.24 hours in apple cider made from McIntosh apples to a high of 1.02 hours in cider made from Red Delicious apples.

B. fulva ascospores were resistant to DMDC up to 1000 mg/L concentration in apple juice, whereas only 25 to 75 mg/L was required to kill the vegetative cells. Increased effectiveness was noted at higher treatment temperatures (Van der Riet et al., 1989).

Martienssen (1961) found DEDC could be used to stabilize fruit juice and prevent fermentation for several days. She suggested that syrups with more than 50% sugar would delay the hydrolysis of the ester. Mehltitz and Gierschner (1964), who investigated DEDC sterilization failures, noted that possible mold contamination points, such as corks, had to be sterilized before treatment of juices with DEDC. Wucherpfenning (1966) pointed out that the DEDC reacts with many components in the juices and therefore the juice can no longer be considered "natural." Attempts to use DEDC to prevent malolactic fermentation in apple juice have met with little success (Hara and Otsuka, 1967). Genth (1969) has reviewed the use of DEDC for cold sterilization of fruit juices.

GRAPE JUICE AND WINES

The effectiveness of DEDC as a sterilant, particularly in grape juice and wines, has been reviewed by Rankine (1964), Pauli and Genth (1966), Pauli (1969), Genth (1971), Barnick (1973), and Ough (1978). Beuchat (1976) investigated the ability of DEDC to inhibit the growth of *Byssochlamys nivea* ascospores in grape juice. This microorganism is known to produce the toxin patulin. It took 500 mg/L of DEDC to inhibit growth of the spores.

Fermentative spoilage of grape juice at 21°C was prevented by addition of 0.8 mM DMDC when juice was inoculated with *S. cerevisiae* Montrachet at levels of 2 or 200 CFU/mL (Terrell et al., 1993). These researchers also noted that DMDC was generally more effective than sulfur dioxide and sorbic acid and that effectiveness of DMDC, but not the other chemicals, was enhanced when the storage temperature was increased to 31°C. Similarly, Morris et al. (1996) demonstrated that 0.8 mM DMDC effectively prevented spoilage of grape juice held at 31°C, whereas sorbic acid, potassium metabisulfite, and combinations of these two delayed but did not prevent spoilage.

The initial work outside the Bayer AG laboratories was reported by Hennig (1959, 1960) and indicated that DEDC was a satisfactory inhibitor and had active cidal properties. He indicated that the inhibitory effect required about one third the amount of DEDC, as did the killing effect. Hennig (1961) also developed the atomizing pump system that came into general use for application of the compound. Mayer and Luthi (1960) also did some of the initial testing on wine in Switzerland and suggested 50 to 100 mg/L of DEDC as an effective sterilizing level. Ough and Ingraham (1961) confirmed that 50 to 100 mg/L of DEDC was a safe sterilizing level in wine and also noted no identifiable sensory problems with DEDC. A sensory threshold for DEDC was found at about 600 mg/L (Van Zyl, 1962; Blouin and Barthe, 1963). Van Zyl (1962) also noted the synergetic effect of DEDC with sulfur dioxide.

Italian studies by Fontana and Colagrande (1964) indicated that levels of 150 to 200 mg/L DEDC were required for effective sterilization. This was further verified for both wine and grape juice by Garoglio and Stella (1964) and Minarik (1964) in Czechoslovakia, Adams (1965) in Canada, Kalugina (1965) in Russia, Todor et al. (1967) in Romania, Ivanov et al. (1967) in Bulgaria, Prillinger (1964) in Austria, and Rankine and Pilone (1974) in Australia. All these researchers tested DEDC in a practical manner, and most determined it to be a very effective antimicrobial agent for use in bottling wine or grape juice in conjunction with the use of good standard sanitary practices. These are only a representative group of the published reports.

Relatively large amounts of ethyl urethane (up to 600 mg/L) were found in a number of commercial Japanese sakes (Ough, 1978). No diethyl carbonate was found in these samples, indicating that no ethanol was present when the DEDC was used. It appears that this method was used commercially in Japan to sterilize the koji before the alcoholic fermentation (Hara et al., 1970). Further studies (Ough et al., 1988a) showed that heating of ethyl alcohol and urea in water solutions resulted in formation of ethyl carbamate. In wine, Ough et al. (1988b) found urea when formed during fermentation could remain in the wine. If the wine was heated, ethyl carbamate formed. Freshly made sake has no more urea than any other similar ferment; however, in the processing of sake, it is heated. This results in significant amounts of ethyl carbamate.

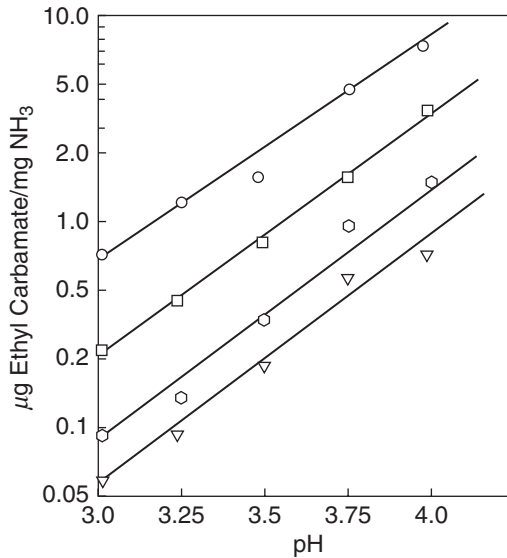


FIGURE 10.2 Influence of ammonia, pH, and DEDC (diethyl dicarbonate) on the amounts of ethyl urethane formed, mg/L of DEDC: 50 (triangles); 100 (hexagons); 200 (squares); 400 (circles).

SOFT DRINKS

DEDC appears to have some value in inhibiting growth of osmophilic yeasts in natural fruit juice soft drinks (Pozsonyi, 1972). A maximum of 300 mg/L of DEDC was suggested for use in sterilizing the drink against yeast or bacterial growth (Pozsonyi, 1972). Molds were not found to be sufficiently killed or inhibited. Pátkay et al. (1973) investigated the use of DEDC with various diluted and carbonated commercial syrups and found that a level of 200 mg/L of DEDC was sufficient to protect the drinks.

DMDC has been suggested for commercial use in Germany as a sterilant for artificial drinks (Genth, 1980). The advantage of using DMDC is that no reactions occur with sugar, sugar alcohols, or artificial sweeteners, such as saccharin or cyclamate.

FOODS

Rash (2003) reported that soaking cantaloupes for 3 minutes in a 10,000-ppm solution of DMDC reduced the population of *Salmonella* from 5.01 log cfu/cm² to undetectable levels; *Salmonella* populations were detected only by enrichment after a 3-minute treatment with 5000 ppm DMDC. Mount et al. (1999) demonstrated that shelf life of fresh salsa could be extended by several weeks by a combination of 100 ppm DMDC and 1% potassium sorbate. They reported that the aerobic plate count and yeast/mold count of fresh salsa decreased from 6 and 2 log CFU/g, respectively, to undetectable levels within 8 days of storage at 4°C.

Molin et al. (1963) summarized the possible uses of DEDC in foods. They found that the growth of *B. cinerea* on strawberries was delayed by dipping the fruit into 100 and 1000 mg/L solutions of DEDC. No adverse sensory effects of the DEDC were noted. In testing apple sauce, they noted that up to 1000 mg/L was required to inhibit mold growth and was not very effective in preventing fermentation of apple sauce.

The storage life of freshly slaughtered chicken carcasses dipped into a solution of DEDC by Schmidt-Lorentz (1962) was extended by 65%. Surface bacteria showed a 10- to 20-fold reduction.

Hara and Otsuka (1966a) studied the use of DEDC to sterilize koji. They determined the most satisfactory approach was to mix the koji in water and then add DEDC. The wild yeast and some bacteria present were killed by 100 to 200 mg/L, but the lactic bacteria and *Aspergillus oryzae* required more than 600 mg/L of DEDC.

BEER

Mönch (1961) appears to have been the first to test DEDC in beer sterilization. His studies indicated that levels between 250 and 1000 mg/L were required for effective action against spoilage by yeast. He noted a taste threshold in beer of 1000 mg/L of added DEDC. Molin et al. (1963) indicated that 0.01% DEDC doubled the shelf life of a beer. A system was suggested by Kozulis et al. (1971) in which DEDC was used in conjunction with *p*-hydroxybenzoate esters for beer sterilization.

TOXICOLOGY

Because the dicarbonates are expected to be decomposed before consumption, the reaction products are of major concern when considering their use in foods. Direct consumption or contact with the material, however, must not be ignored.

FOODS

Because DMDC is hydrolyzed to carbon dioxide and methanol almost immediately after addition to beverages, the FDA determined that there would be virtually no exposure of consumers to the additive itself when used within the limits of 200 ppm in beverages (FDA, 1988). Methanol is the principal hydrolysis product of concern resulting from addition of DMDC to wine. Theoretically, complete hydrolysis of DMDC would yield 2 moles of methanol from 1 mole of DMDC added to wine. Using a worst-case scenario, the FDA determined that consumption of wine containing 200 ppm DMDC at a rate of 232 g per person per day would result in a daily intake of not more than 22 mg per person per day, well within safe limits (FDA, 1987). An adult human can metabolize up to 1500 mg of methanol per hour with no adverse symptoms or effects (Lehman, 1963). A fairly extensive report of the safe use of DMDC in wine, including reference to breakdown and reaction products, is described in the FDA response to the original DMDC food additive petition (FDA, 1988).

Hecht (1961) found no significant toxic effects in rats consuming DEDC-treated grape juice for a 59-day period. This observation was confirmed by Bornmann and Loeser (1961). Although DEDC-treated milk caused some weight loss, no other symptoms were detected by these workers.

Bornmann and Loeser (1966) did long-range studies using the reaction product of DEDC and ethanol (diethyl carbonate, DEC). No harmful effects were found when DEC was given to rats at 0.3% in drinking water for 100 weeks or by force feeding it to dogs at 600 mg/kg per day for 3 months.

Sharratt et al. (1972) repeated the short-term tests using wine, beer, orange juice, and black currant juice. Treatments with DEDC caused no adverse effects from the reaction products on the rats being tested.

Studies by Lang et al. (1966) showed that most of the reaction products were hydrolyzed enzymatically to the original compound plus carbon dioxide and ethanol. The exception was the carbethoxy ascorbic acid. This product hydrolyzed into the original ascorbic acid, dehydroascorbic acid, diketogluconic acid, and furfural after a few hours. Paulus and Lorke (1967) tested these products in short-term studies on rats and found no adverse effects. Wolf et al. (1969) found no toxic effects in five human volunteers who over a 40-day period ingested 250 mL wine per day that was treated with 150 mg/L of DEDC. A single treatment of 1 L of the treated wine caused no

toxic symptoms in the group. Zaitsev et al. (1970) suggested that DEDC not be used in foods because of its toxicity. Their fear was that the compound would not completely hydrolyze.

Santini et al. (1985) tested the mutagenicity of DEDC on two strains of *Salmonella* Typhimurium (His⁻) and on *Bacillus subtilis* 170 (Trp⁻, Ura⁻) and found no reversion to prototrophy. They also found no effect of ethyl carbamate on the mutagenicity of *B. subtilis*. There was some transformation reduction.

The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (1972) studied the use of DEDC and concluded that beverages over pH 4.0 with a significant content of ammonia, amino acids, or proteins (e.g., milk) should not be treated with DEDC. Treatment of beer and fruit juices is not technically justified, nor should wine be treated with DEDC. The acceptable level of treatment for soft drinks, carbonated or not, is 250 mg/L. Under no circumstances should the human daily intake of ethyl carbamate exceed 100 µg/day. No position has been taken on DMDC by the FAO/WHO.

Short-term toxicologic studies reported by Genth (1980) indicated the acute toxicity for rates of DMDC were 330 to 900 mg/kg body weight. The Ames test for mutagenicity was negative. No adverse effects were found after feeding fruit juice and alcoholic beverages treated with 4000 mg/L of DMDC for 3 months. The amounts of methanol produced were well below toxicologic levels. Methyl urethane, another reaction product, was reported not to be a carcinogen (Pound, 1967).

DIRECT EXPOSURE

The median lethal dose (LD₅₀) values of DEDC for mice, rats, cats, rabbits, and dogs were reported to be 1558, 850, 100 to 250, 500 to 750, and greater than 500 mg/kg body weight, respectively, by the oral route (Joint FAO/WHO Expert Committee on Food Additives, 1972). Tests with rabbits, guinea pigs, rats, and mice (Hecht, 1961) showed 1 mg/L exposure in air caused chronic respiratory symptoms and 10 mg/L was lethal. It is a strong skin and eye irritant.

INDIRECT EXPOSURE

The carcinogenicity of ethyl carbamate has been well documented in animals (Allen et al., 1986; Miller and Miller, 1983; Mirvish, 1968; Schmähl et al., 1977; Woo, 1983).

Uzvolgyi et al. (1983) found that DEDC and ammonia incubated *in vitro* in rat gastric juice and then administered to mice orally caused lung cancer in the mice. If DEDC and ammonia were administered by probe *in vivo* to mice, the same effect was found. Later Uzvolgyi (1986) reported contrary results when testing lactating mice and their offspring.

ANALYSIS

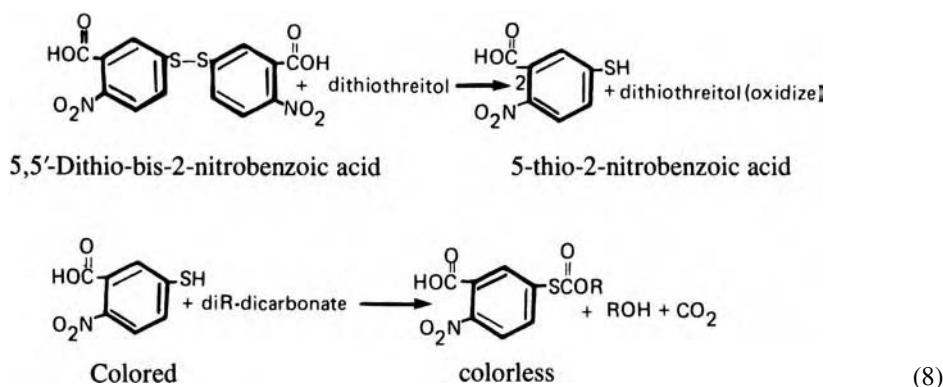
Both DEDC and DMDC are very reactive, and if any water or other proton donor substance is present, they break down autocatalytically. Two types of analyses are available: one to measure the pure or nearly pure substance and the other to measure the residual amount of a specified reactant in the treated material and calculate the amount of dicarbonate added. The first is used primarily to determine the purity of the dicarbonate or to study the kinetics of its reaction. The other method is used at some later date to determine the amounts added originally.

DIRECT

The dicarbonates, either diethyl or dimethyl, can be determined. DEDC was measured with a relative standard deviation of ±0.3% by Cuzner et al. (1971). A measured amount of dicarbonate was combined with an excess but known amount of morpholine. The morpholine reacted with the dicarbonate. The remaining morpholine was titrated with standardized methanolic HCl solution to a pH endpoint of 4.0. Hara and Otsuka (1966b) tested three other amines besides morpholine and

determined that *n*-butylamine and isobutylamine gave the sharpest endpoint. They extracted dicarbonate from sake with organic solvents before analysis. Monceli (1970) has suggested a spectrophotometric method using 4-aminoantipyrine, which requires an extraction step.

Peterson (1978) used the method suggested by Berger (1975) for his studies on hydrolysis rate. This spectrophotometric method lends itself to multisample analysis. The reactions involved in this method are as follows:



The colored compound 5-thio-2 nitrobenzoic acid is destroyed by the dicarbonate. Pseudo-first-order kinetics describe the reaction. The rate of the color disappearance is proportional to the initial amount of dicarbonate present.

INDIRECT

The reactions of DEDC with alcohols were postulated earlier. Kielhöfer and Würdig (1963a,b) were first to use the formation of diethyl carbonate to measure the original amount of DEDC added to an alcoholic beverage. They found no diethyl carbonate (DEC) in untreated wine. The wines treated with DEDC formed DEC in amounts proportional to the amount of ethanol present in the wine. The method consisted of extraction of the wine with carbon disulfide or pentane and determining the DEC by gas chromatography. The amounts found are in the range of 5 to 10 mg/L of DEC in most wines treated with the usual amount of DEDC. Prillinger (1964) verified the earlier work. Garschagen (1967) found that DEC could be measured by gas chromatography directly without extraction. A very sensitive headspace method that could detect DEC to 0.1 mg/L was suggested by Kunitake (1969).

Wunderlich (1972) reported a collaborative study for the Association of Official Analytical Chemists. The method involved extracting the DEC and determining the amount with quantitative gas chromatography. McCalla et al. (1977) questioned the use of the 15% Carbowax 20-M column used for the separations. They found an unknown peak at the same retention time as DEC. Using headspace sampling and chromatography coupled to a mass spectrometer, Van Lierop and Nootboom (1979) were able to measure as little as 1 µg/L of DEC. The specificity of measurement was enhanced when the mass spectrometer was selectively tuned to direct M/E 63 and 91, the main fragments of DEC. The official method (Horwitz, 1980) is extraction with carbon disulfide, separation on the gas chromatographic column, and comparison of the DEC peak area with nonalcoholic foods or beverages.

The DMDC reaction product with ethanol is ethyl methyl carbonate (EMC). Stafford and Ough (1976) reported a method to detect the amount of EMC formed and to relate it to the amount of DEDC added to an alcoholic beverage. They used a gas chromatographic method similar to that of Wunderlich (1972) for DEC measurement but used an internal standard (DEC) to allow more

accurate determination. Bandion et al. (1979) reported a similar determination using glass capillary columns for better separation.

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11 Medium-Chain Fatty Acids and Esters

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CONTENTS

Antibacterial Fatty Acids	328
Antifungal Fatty Acids.....	330
Chemical Modifications of Fatty Acids.....	331
Fatty Acid Structure–Function Activity.....	332
Antimicrobial Fatty Acid Monoesters	333
Glycerol Monolaurate (Monolaurin)	336
Mechanism of Action.....	346
Future Applications	348
References	352

The word preservative on food product labels seems to evoke a negative reaction from many consumers. Although consumers want natural, fresh products, the ability of the food industry to deliver foods to the marketplace usually depends on adding chemicals to lengthen shelf life. The phrase “chemical additive” does not always mean unnatural or unsafe. One group of chemicals found in nature and considered to have little or no toxicity is the fatty acids and their corresponding esters. Indeed, early records suggest that fatty acids have a long and respected historical record for having antimicrobial activity. In fact, one of the nutritional factors in human milk that both aids infant growth and development (energy source) and acts as a natural antimicrobial is the high level of lauric acid fats. Lauric acid fats provide up to 12% of total fat content in milk and 3.5% to 6.6% of milk calories (Chen et al., 1996). Another high lauric acid fat-containing food is coconut.

Soaps (salts of fatty acids) have long been used as cleaning and disinfecting agents. On a 4000-year-old clay tablet uncovered at Tello, Mesopotamia, soap was mentioned. Soap was well known to “barbarous” people, rather than being the discovery of “civilized” groups. The Gauls and Germans used soap before the Christian era. Soap came to the Romans before the year 79 CE (common era), when Vesuvius overwhelmed Pompeii. From the Romans, the Greeks learned the value of soap. The pharmacologic use of soaps followed its detergent uses. Its germicidal value was probably recognized much earlier than records show. The use of soap as an insecticide was recommended by Goeze in 1787. Much of the literature of soap as an insecticide can be found in a marvelous old monograph (Shepard, 1939). In more recent history, the earliest mention of soap as an antifungal agent can be found in a paper by Clark (1899). Another early worker, Reichenback (1908), studied the action of soaps on *Escherichia coli* and found that the potassium salts of myristate, palmitate, and stearate were effective against this bacterium, but oleate, erucate (Δ^9 -C22:1), and linoleate were inactive.

Much of the work carried out on the germicidal activity of soaps can be found in the literature between 1920 and 1940 (Bayliss, 1936; Kodicek, 1949; Nieman, 1954; Kabara et al., 1977). Before 1930 the lack of success in the search for active antibacterial agents other than fatty acids evolved

a pessimism regarding whether an active agent would ever be found. Two important discoveries changed the course of thinking on the subject. First was the serendipitous finding of Alexander Fleming, who in 1928 observed the lysis of *Staphylococcus* colonies in an area surrounding the growth of a *Penicillium* mold, and thus antibiotics were added to the small arsenal of compounds lethal to microorganisms (Fleming, 1929). Second, the discovery of antimicrobial sulfanilamides in 1935, first observed by Trefouel, Nitti, and Boret in Fourneau's laboratory (Pasteur Institute), gave hope for finding new and more effective synthetic biocidal agents (Mellon et al., 1939). Thus, these two major discoveries in less than a decade gave tremendous impetus to the search for still more active compounds. The work being carried out with fatty acids was to suffer because the biocidal activity of the isolated antibiotics and synthesized germicides was so much greater than that of fatty acids. In proportion to the speed with which new and more powerful drugs were discovered, interest in the action of fatty acid biocides waned. The frantic research activities in the 1950s and 1960s produced hundreds of antibiotics and synthetic products useful to the pharmaceutical and cosmetic field but of little use to the food industry. It is interesting that the food industry kept distant from these new discoveries for the most part and continued to use old standbys.

Several reasons can be given for the "nonparticipation" of the food microbiologist. First, antibiotics and synthetic germicides were not without health risks in terms of toxicity and the possible development of resistant strains; second, the cost of these newer agents precluded their use in quantities normally required in the food industry; and third, the use of old standbys was sufficient (Lueck, 1980). Standbys included common salt and smoke from prehistoric times, vinegar from ancient Egypt, sorbic acid isolated from rowanberry oil by Hoffman (1859), benzoic acid discovered by Fleck (Strahlmann, 1974), and propionic acid recommended for the preservation of bakery goods by Watkins (1906). Despite the lack of interest in food applications, it is obvious that some fatty acids have played an important historical role in food preservation. Many other fatty acids, even more active than the standbys, have not been fully examined as potential preservatives, nor have detailed studies dealing with their structure-function relations been carried out. To collate and evaluate the literature dealing with this problem, the following review of antimicrobial fatty acids and their corresponding esters is presented.

ANTIBACTERIAL FATTY ACIDS

Walker (1924, 1925, 1926) showed that sodium and potassium soaps of the same acid did not vary greatly in their germicidal action. The lower members of this series, 4 to 19 carbon chain lengths (C₄ to C₁₉), had little or no germicidal effect. He found pneumococcus to be very susceptible to laurates, oleates, linoleates, and linolenates. Streptococci were killed much like pneumococci but at higher concentrations of soap. Hettche (1934) found that unsaturated soaps, such as oleate, linoleate, and linolenate, were germicidal toward *Staphylococcus* but not toward a colon bacillus.

Larson (1921) found that pneumococci and streptococci would not grow in the presence of even small amounts of sodium ricinoleate. Larson and Nelson (1925) reported that pneumococci instantly lost their pathogenicity on treatment with castor oil soap at a final dilution of 0.1%. Scarlet fever streptococci lost their power to grow after 5 minutes in 0.5% sodium ricinoleate. Miller and Castles (1931) found the same fatty acid inhibited the growth of gonococci on artificial media in dilutions of 1:20,000. Violle (1933) studied the effect of a 1:1000 solution of fatty acids on many kinds of bacteria. The common and pathogenic bacteria of the intestinal tract were unaffected. Streptococci were killed, but staphylococci were not. Barnes and Clarke (1934) determined that 0.004% sodium ricinoleate and 0.004% sodium oleate were approximately the minimum inhibitory concentrations (MICs) of the soaps against three types of pneumococci. In other words, oleate was more effective by a factor of 10. Kolmer et al. (1934) reported that a 20% solution of sodium ricinoleate was completely bactericidal for *Staphylococcus aureus* after exposure for 5 minutes, yet a 10% solution was not completely bactericidal for exposures as long as 1 hour when tested according to the Reddish method.

Bayliss (1936) studied the relation between the structure of soaps and their germicidal properties. A comparison of the pneumococcidal properties of the saturated soaps, laurate, myristate, palmitate, and stearate, showed that the soap containing 14 carbon atoms had the maximum antimicrobial activity. In some cases, the presence of unsaturated bonds in the molecule tremendously altered soap activity against pneumococci. For example, sodium oleate (18 carbon, 1 double bond) was greater than 100 times more effective than either sodium stearate (18 carbon, no double bonds) or phenol in destroying pneumococci. The addition of one, two, or three more double bonds, as in the 18 carbon oleic, linoleate, or linolenate soaps, respectively, caused no change in this property. However, the two soaps, sodium α - and β -elaeostearates, although isomeric with sodium linolenate, were relatively ineffective as pneumococcicides. The three unsaturated soaps demonstrated pneumococcidal properties considerably weaker than those of sodium oleate, and so on. Ricinelaideate, a geometric isomer of sodium ricinoleate that has physical properties much like stearate and palmitate, was somewhat less effective than ricinoleate. Ricinstearolate, containing a triple bond instead of a double bond and with physical properties very similar to those of ricinoleate, is even less active against bacteria. Sodium undecylenate, containing 11 carbon atoms and a double bond at the end of the hydrocarbon chain, required a fairly high concentration to destroy pneumococcus.

The effect of soaps on lactococci, which cause souring of milk and are resistant to disinfection, did not parallel the effect of the same soaps on pneumococci. In no case, however, did lactococci require lower concentrations to kill than to kill pneumococci. Of the saturated soaps, only myristate and laurate exhibited killing action on *Lactococcus lactis*. The addition of one double bond, as in sodium oleate, gave the soap the ability to kill this bacterium in concentrations equal to that required by sodium laurate and myristate. The addition of a second double bond, as in linoleate, enhanced this killing property considerably. Further addition of a third double bond, however, conferred no greater ability to kill lactococci. Cowles (1941) showed that at low pH values fatty acids have bactericidal action and that their activity increases with chain length. Cells of *E. coli* became more sensitive to medium chain fatty acids after exposure to sublethal heat stress (Tsuchido and Takano, 1988). Several meat spoilage bacteria were tested for their sensitivity to saturated and unsaturated fatty acids (C12 to C18) (Ouattara et al., 1997). *Brochothrix thermosphacta*, *Pseudomonas fluorescens*, and *Serratia liquefaciens* were not inhibited by any of the fatty acids but *Carnobacterium piscicola*, *Lactobacillus curvatus*, and *Lactobacillus sake* were inhibited most by lauric and palmitoleic acids and less so by linoleic and linolenic acids. *Carnobacterium piscicola* was the most susceptible to inhibition among the bacteria tested.

Kodicek (1949) tested a number of fatty acids on *Lactobacillus casei* and found that in the saturated series lauric acid depressed growth, but the effect was not reversible by cholesterol. The inhibition by other fatty acids, including the more active and branched-chain α -methyl lauric acid, could be reversed by addition of the sterol. As found by Bayliss (1936), Kodicek (1949) found that of the unsaturated series, activity was limited to *cis* isomers rather than *trans* isomers. Similar bacteriostatic effects were observed against *S. aureus* (Hettche, 1934), *Glaucoma piriformis* (Chaix and Baud, 1947), and *Mycobacterium tuberculosis* (Bergstrom et al., 1946; Dubos and Davis, 1946, 1947). The evidence of the action of unsaturated fatty acids on bacteria *in vitro* helped account for the known biocidal activity of some biological materials. For example, Humfeld (1947) found an antibiotic fatty acid in wheat bran, and McKee et al. (1947) found antibacterial lipids from *Tetrahymena geleii*. Subsequent findings have documented numerous other antimicrobial lipids in natural products. For example, a new antimicrobial fatty acid that was active against Gram-positive bacteria was extracted from moss (Borel et al., 1993). The compound dicranin (9,12,15-octadeca-trien-6-ynoic acid) was active against several bacilli and against *S. aureus* and *Enterococcus faecalis*. Medium-chain fatty acids (lauric acid and myristic acid) produced by blue-vein cheese molds were found to inhibit growth of *Listeria monocytogenes* (Kinderlerer et al., 1996).

It has been well established that unsaturated fatty acids have antibacterial influence on Gram-positive bacteria. The subject was reviewed by Nieman (1954), who concluded that the inhibitory

effects of unsaturated fatty acids increased as the number of double bonds in the molecule increased. Thus, the inhibitory effect of linoleic acid was far greater than that of oleic acid (Fuller and Moore, 1967). However, the MIC of arachidonic acid was about the same as that of linoleic acid, whereas the MIC of linolenic acid was somewhat greater than that of linoleic acid.

ANTIFUNGAL FATTY ACIDS

The antifungal activity of fatty acids has been recognized for many years (Clark, 1899; Kiesel, 1913; Wyss et al., 1945). The preceding authors and others (Kitajima and Kawamura, 1931; Tetsuomoto, 1933a, b; Thornton, 1963; Kodicek and Worden, 1945) demonstrated that the fungitoxicity of these compounds was dependent on chain length, concentration, and pH of the medium. Several materials can act as protective agents against the antimicrobial action of the fatty acids. These antagonistic substances include serum albumin, starch, cholesterol, lecithin, saponin, and charcoal (Nieman, 1954). Although a completely satisfactory explanation of the mode of action of these compounds has not yet been presented, the evidence (Tetsuomoto, 1933a) seems to indicate that growth inhibition is the result of an alteration in cell permeability (Kodicek and Worden, 1945). As systemic antifungal agents, the fatty acids, despite their low order of toxicity, have not been effective. This may be because they are readily metabolized by the fungus through the usual fatty acid pathways. In general, they may be esterified to form glycerides and/or may be degraded to small fragments by oxidation.

Asami et al. (1965) demonstrated that 11-iodo-10-undecenoic acid was esterified, in part, by the rat to a glyceride. Although the effect of fluorine in the 2-position of fatty acids on esterification has not yet been reported, these fatty acids are believed not to undergo oxidation (Pattison et al., 1965). Thus, 2-fluoro fatty acids possess at least one potential advantage over the nonfluorinated analogs that would be useful for systemic antifungal activity (Gershon and Parmegiani, 1967). The antifungal activity of several 2-fluoro fatty acids up to a chain length of 20 carbon atoms was determined in parallel with the corresponding nonfluorinated analogs against four fungi: *Aspergillus niger*, *Trichoderma viride*, *Myrothecium verrucaria*, and *Trichophyton mentagrophytes* (Gershon and Parmegiani, 1967). Both series of compounds were about equally active, except that the nonfluorinated fatty acids showed maximal activity at chain lengths of 4 to 10 carbons, whereas the 2-fluoro fatty acids were most active at chain lengths of 8 to 14 carbons. Because the pKa values of the 2-fluoro fatty acids are generally two units lower than those of the nonfluorinated fatty acids, it appears that the pKa of the fatty acid does not play an important role in antifungal activity.

Interest in controlling dermatomycoses (fungal skin infections) has suggested further investigation of the reported activity of fatty acids and related compounds as antimycotic agents may be warranted. This problem was first studied by Clark (1899), who reported the effect of acids, including fatty acids, on the germination of fungi. Work by Kiesel (1913) at the Pasteur Institute revealed many of the facts known today about the characteristics of the antimycotic action of fatty acids. He found the following:

1. Activity of saturated fatty acids increased as the number of carbon atoms in the fatty acid chain increased up to 11 carbon atoms.
2. Branched-chain fatty acids were less active than those with straight chains and an equal number of carbon atoms.
3. Substitution of hydrogen by hydroxyl decreased activity.

Japanese workers (Kitajima and Kawamura, 1931; Tetsuomoto, 1933a, b) confirmed Keisel's work but found that with the saprophytic wood-rotting fungi the optimum activity was with the 12-carbon acid. Unsaturated acids were found to be more active than the corresponding saturated acids, and dicarboxylic acids were almost without activity. Other efforts provided further confirmatory evidence using different conditions and different microorganisms (Kirby et al., 1937; Peck

and Rosenfeld, 1938; Peck et al., 1939; Baechler, 1939; Hoffman et al., 1939, 1940; Rigler and Greathouse, 1940). Keeney (1943, 1944) reported fungicidal activity for the long-chain acids but much less or no killing action by the short-chain members of the fatty acid series. Data showed that the optimum chain length was species dependent and was affected by the solubility of the fatty acids in question. Thus, for *A. niger* the optimum chain length was 11 carbon atoms, but for *Trichoderma interdigitale*, the 13-carbon acids were most active. *Trichoderma purpureum* was even less resistant and was inhibited by 14-carbon acids, suggesting that longer chain length compounds failed to show antimycotic effects because their low solubility prevented activity. As with bacteria, the data showed that the fatty acids increase in antifungal activity with decreasing pH, provided that the low pH values do not make the compound so insoluble that a static concentration for the organism under test cannot be obtained. The change in activity with hydrogen ion concentration was much greater for the short-chain acids, suggesting that the ion of the long-chain compound — or some aggregate or micellar form of it that may exist in solution — exerts additional action.

CHEMICAL MODIFICATIONS OF FATTY ACIDS

Eggerth (1926, 1927, 1929, 1931) found that α -bromo fatty acids were usually more germicidal than unsubstituted soaps. The effect of a hydroxyl in the α position was to increase the selective germicidal action of saturated soaps and to diminish that of unsaturated soaps. The hydroxylated salts, gluconate and trihydroxystearate, seem to lack any ability to kill pneumococci under the conditions used (Bayliss, 1936). Stearate is peculiar in that the addition of a 4-hydroxyl group restored the power to destroy these bacteria in fairly low concentrations. Hydroxylation, in the case of the ricinoleate, enhanced the bactericidal activity against streptococci, whereas there was a decrease in activity against pneumococcus. The replacement of the ethylenic linkage in fatty acids by an acetylenic linkage caused a marked decrease in germicidal activity (Bayliss, 1936). Sodium abeitate, which is an alicyclic soap, had destructive action on pneumococcus at one tenth the concentration necessary for phenol. The same was true for chalmaugrate, which has been used as a chemotherapeutic agent in the treatment of Hansen's disease (Bayliss, 1936). The introduction of a 6-phenyl group on an 11-carbon acid decreased antimicrobial activity markedly, regardless of the degree of unsaturation of the fatty acid chain. Substitutions in the phenyl ring with an amino or hydroxyl group on the fatty acid chain resulted in activity comparable to that of the corresponding straight-chain acids. No variation that has been made in the arrangement of the carbon atoms has brought about greater activity than that displayed by the straight-chain acids (Bayliss, 1936).

Since early times, sulfur has been considered efficacious in the treatment of fungal diseases. Because a sulfur atom is considered equivalent to $-\text{CH}=\text{CH}-$, n-heptylmercaptoacetic acid may be regarded as the isostere of an unsaturated 11-carbon fatty acid. Neither this compound nor a variety of other substituted mercaptoacetic acids showed activity of the same magnitude as that of the fatty acids (Bayliss, 1936). With the exception of 11-thiohendecanoic acid, none of the other thiohendecanoic acids approached undecylenic acid in activity. The latter fatty acid was found by Rothman et al. (1945) to be responsible for the spontaneous cure of ringworm of the scalp in puberty.

Branching of the carbon chain decreases activity if one considers the total number of carbon atoms in the acid. The effect of branching is less, however, when one considers only those carbon atoms in the longest chain. Fatty acids substituted with aldehyde, acetate, ethyl ester, amide, or substituted amide have considerable activity but are less active than the corresponding acid. Alcohols have high activity, but owing to their limited solubility, the effectiveness of long-chain alcohols can be demonstrated only on more sensitive microorganisms (Kabara et al., 1972). A novel chemical alteration of the antibacterial enzyme lysozyme by covalent bonding with palmitic, myristic, or stearic acids increased the spectrum of activity of the hydrolytic protein to include both Gram-positive (native protein) and Gram-negative bacteria (Ibrahim et al., 1991, 1993).

TABLE 11.1
Minimum Inhibitory Concentrations of Fatty Acids against
***Clostridium perfringens*^a**

Fatty Acid	Minimum Inhibitory Concentration (mg per 100 ml)	
	pH 6.5	pH 7.5
Caproic (6:0)	1160	5810
Caprylic (8:0)	721	3610
Capric (10:0)	172	862
Lauric (12:0)	1000	200
Myristic (14:0)	2280	457
Palmitic (16:0)	2560	2560
Stearic (18:0)	2850	2850
Oleic (18:1)	283	283
Erucic (22:1)	339	339
Linoleic (18:2)	5.61	5.61
Linolenic (18:3)	27.90	27.90
Arachidonic (20:4)	6.09	6.09
Methyl linoleate	146	733
Methyl linolenate	293	1460

^a Fatty acids and methyl esters in 0.1 M phosphate buffer (pH 6.5 or 7.5) containing 0.1% (wt/vol) Tween 80.

Source: Tetsumoto (1933b).

FATTY ACID STRUCTURE–FUNCTION ACTIVITY

Many authors have discussed the antimicrobial effects of fatty acids, and the consensus is that their action is a result of the undissociated molecule (protonated acid), not the anion (Winslow and Lochridge, 1906; Reid, 1932; Hoffman et al., 1939; Levine and Fellers, 1940; Albert, 1960). If this is so, their activity should be profoundly affected by pH because this determines the degree of dissociation of the acid. Indeed, this fact has been shown, where more rapid killing occurs at lower pH (Meynell, 1955; Prince, 1959). An example of this finding is shown for *Clostridium perfringens*, where the MICs of selected fatty acids are given in Table 11.1. An increase from pH 6.5 to 7.5 increased the MICs of the short-chain acids (caproic, caprylic, and capric) but decreased the MICs of the two medium-chain fatty acids (lauric and myristic). The MICs of the unsaturated fatty acids were unaffected by a change in pH in the medium. Of the fatty acids tested, those with the lowest MIC were the polyunsaturated acids, such as linoleic, linolenic, and arachidonic acids. However, the MIC of linoleic acid, with two double bonds, was somewhat less than that of linolenic acid, with three double bonds.

In an attempt to bring some order to the vast amount of literature on structure–function activity, a series of studies were carried out with very pure (99%) fatty acids and derivatives. The results in Table 11.2 and elsewhere (Kabara et al., 1972, 1973; Conley and Kabara, 1973; Kabara, 1982) are the basis for a number of generalizations that help to explain most of the data in the literature:

1. Except for short-chain fatty acids (fewer than 8 carbon atoms), lipids do not affect Gram-negative bacteria.
2. The most active chain length for saturated fatty acids is C12; the most active monounsaturated fatty acid is C16:1, and C18:2 is the most active polyunsaturated fatty acid.

TABLE 11.2
Minimum Inhibitory Concentrations of Saturated and Unsaturated Fatty Acids^a

Fatty Acid	Pneumococci	<i>Streptococcus</i> Group A	<i>Streptococcus</i> χ -Hemolytic Non-A	<i>Candida</i> <i>albicans</i>	<i>Staphylococcus</i> <i>aureus</i>
Caproic	NI ^b	NI	NI	NI	NI
Caprylic	NI	NI	NI	NI	NI
Capric	1.45	1.45	2.9	2.9	2.9
Lauric	0.062	0.124	0.249	2.49	2.49
Myristic	0.218	0.547	2.18	4.37	4.37
Myristoleic	0.110	0.110	0.110	0.552	0.441
Palmitic	0.48	3.9	3.9	NI	NI
Palmitoleic	0.024	0.098	0.049	0.491	0.983
Stearic	NI	NI	NI	NI	NI
Oleic	NI	1.77	NI	NI	NI
Elaidic	NI	NI	NI	NI	NI
Linoleic	0.044	0.089	0.089	0.455	NI
Linolenic	0.179	0.35	0.35	NI	1.79
Linolelaidic	NI	NI	NI	NI	NI
Arachidonic	NI	NI	NI	NI	NI

^a Results are given in mM.

^b NI, not inhibitory at the concentrations tested (1.0 mg/ml or 3.0 to 6.0 mM).

3. The position and number of double bonds are more important to long-chain (>C12) than shorter chain (<C12) fatty acids.
4. The *cis* form is active; the *trans* isomer is inactive.
5. Acetylenic derivatives, compared to ethylenic fatty acids, are more active against fungi.
6. Yeasts are affected by fatty acids with short chain lengths (C10 to C12) — that is, slightly shorter than those affecting Gram-positive bacteria.
7. Fatty acids esterified to monohydric alcohol are inactive; esterification to polyols increases activity.

ANTIMICROBIAL FATTY ACID MONOESTERS

Esterification of fatty acids with a monohydric alcohol (e.g., methanol and ethanol) resulted in esters that were inactive (Kabara et al., 1972). This was not true when an α -hydroxy fatty acid was esterified (Kabara et al., 1972). From these initial results it was concluded that some hydrophilic group was necessary for biological activity and that the esterification of a polyhydric alcohol would yield active derivatives (Conley and Kabara, 1973). The need for free single or multiple hydroxyl groups before biological activity of the ester was thus established. One of the more common polyhydric alcohols, glycerol, was esterified and found to be more active than the corresponding fatty acids (Table 11.3). Details of these findings have been published (Conley and Kabara, 1973; Kabara et al., 1977; Kabara, 1982) and confirmed by others (Kato and Shibasaki, 1975b; Shibasaki and Kato, 1979; Sands et al., 1979; Beuchat, 1980).

There seems to be some controversy or confusion about which fatty acid ester is the most biocidal. As a general statement, the fatty acid used to esterify the polyol determines the potency of the ester. The structure–function relation for saturated and unsaturated esters follows the activity of their respective fatty acids, as reviewed earlier. This means that lauric acid (C12) and palmitoleic

TABLE 11.3
Minimum Inhibitory Concentrations (vg/ml) for Fatty Acids and Their Corresponding Monoglycerides

Microorganism ^a	Undecenoic Acid	10-Undecenoic Acid	10-Undecenoyl Monoglyceride	10-Undecynoyl Monoglyceride	11-Dodecenoic Acid	Dodecanoic Acid	Dodecanoyl Monoglyceride	12-Tridecenoic Acid	Tridecanoic Acid	Tridecanoyl Monoglyceride
<i>Enterococcus faecalis</i>	NI ^b	NI	500	500	NI	500	NI	1000	NI	NI
<i>Streptococcus pyogenes</i>	125	1000	125	125	250	62	8	125	1000	62
<i>Staphylococcus aureus</i>	1000	1000	500	500	NI ^b	500	250	1000	NI	NI
<i>Corynebacterium sp.</i>	31	31	62	62	125	31	16	31	NI	NI
<i>Nocardia asteroides</i>	62	125	125	62	62	62	16	125	1000	125
<i>Candida albicans</i>	1000	1000	250	100	1000	1000	500	1000	NI	NI
<i>Saccharomyces cerevisiae</i>	500	500	250	100	500	1000	250	500	1000	NI

^a *Escherichia coli* and *Pseudomonas aeruginosa* were not affected.

^b NI, minimum inhibitory concentration greater than 1000 µg/ml.

Source: Kabara et al. (1977).

acid (C16:1) form the most active saturated and unsaturated derivatives, respectively (Conley and Kabara, 1973; Sands et al., 1979, Wang and Johnson, 1992). This statement concerning the structure–function relation is true, except when specific microorganisms and food products are concerned because the monoglycerides can be neutralized by a variety of polymers, including starch and protein. Because monoglyceride binding seems to be related to biological activity, several researchers (Ueda and Tokunaga, 1966; Koga and Watanabe, 1968) have reported that volatile short-chain fatty acids esters were more active in foods than higher chain length homologs. These reports must be viewed as exceptions found under particular circumstances. Several other reports have supported the findings that the lauric acid derivative is the most active monoglyceride, even in the presence of food (Beuchat, 1980; Shibasaki, 1982; Wang and Johnson, 1992; 1997). Glycerol monolaurate is hereafter referred to as monolaurin. Potential food applications of monolaurin combined with other monoglycerides have been explored (Wang and Johnson, 1997).

Ababouch et al. (1994) conducted experiments on the ability of fatty acids and monolaurin to inhibit spores and vegetative cells of *Bacillus cereus*. Their work demonstrated that linolenic acid and monolaurin were more effective than lauric, linoleic, oleic, and stearic acids. Monolaurin (0.73 mM), linoleic acid (0.178 mM), and linolenic acid (0.036 mM) inhibited both spore germination and vegetative cell outgrowth. They also found that starch decreased the inhibitory activity of the fatty acids. Spores of foodborne spoilage (*Clostridium sporogenes*) and pathogenic (*B. cereus*, *Clostridium botulinum*) bacteria were generally more sensitive to monoglycerides than are their respective vegetative congener (Chaibi et al., 1996a). The general order of activity from high to low was monolinolenin, monolaurin, monomyristin, and monolinolein. Monopalmitin, monostearin, and monoolein were much less effective against the spore formers. The concentrations of monoglycerides needed to inhibit spore germination and outgrowth were higher than the concentrations needed to inhibit cell multiplication. The main target of inhibition appears to be directed at spore germination and cell growth rather than spore outgrowth (Chaibi et al., 1996b). Monolaurin and monolinolenin are known to lower *B. cereus* heat resistance (Chaibi et al., 1998), which may be useful when lower heating temperatures are desired for commercial sterilization of foods. It is important to note that the manner in which monolaurin is incorporated into test systems will affect its activity (Cotton and Marshall, 1997).

Beuchat (1980) compared the effects of glycerol and sucrose fatty acid esters, benzoate, and sorbic acid and its potassium salt against *Vibrio parahaemolyticus*. His results indicated that the C12 monoglyceride (monolaurin) was more active than lower (C8 or C10) or higher (C14) chain-length derivatives. Also, the low MIC of monolaurin (5 µg/ml) showed it to be more effective than sodium benzoate (300 µg/ml) or sorbic acid (70 µg/ml). Sucrose caprate and sucrose caprylate were found to be effective against *V. parahaemolyticus* but at higher concentrations than monolaurin. Because these esters previously were regarded to be inactive against Gram-negative bacteria (Kabara, 1979a), the inhibition of *V. parahaemolyticus* growth was somewhat surprising, although Kato and Shibasaki (1975a, 1976) earlier reported similar effects on other Gram-negative bacteria but only when chelating acids (citric and polyphosphoric acid) were present. Their interpretation was that chelating acids release a significant amount of cellular lipopolysaccharide from bacterial cell walls and cause the bacterium to behave like a Gram-positive bacterium. Apparently the presence of chelators is not always a prerequisite for demonstrating inhibitory action of monoglycerides against all Gram-negative bacteria because activity against *V. parahaemolyticus* was not chelator dependent (Beuchat, 1980). Combinations of monolaurin with ethylenediamine tetraacetic acid (EDTA), low pH, high salt, and either lysozyme or butylated hydroxyanisole increased activity against a series of Gram-positive and Gram-negative bacteria (Razavi-Rohani and Griffiths, 1996). Monolaurin activity against Gram-negative bacteria was enhanced when combined with EDTA and low pH and/or high NaCl levels (Razavi-Rohani and Griffiths, 1994). Citrate, another cation chelator, was not an effective adjunct to enhance monolaurin activity against Gram-negative bacteria.

The bacterium that causes peptic ulcers, *Helicobacter pylori*, was sensitive to several medium-chain monoglycerides (Petschow et al., 1996). Greatest activity was seen with C10 to C14 monoglycerides, with 1 mM levels resulting in 4-log population reductions after 1-hour exposure. Lauric acid was the only free fatty acid demonstrating activity against this pathogen.

Milk-derived fatty acids and monoglycerides have demonstrated activity against enveloped viruses such as cytomegalovirus, rhinoviruses, and human immunodeficiency virus (HIV) (Welsh et al., 1979; Hierholzer and Kabara, 1982; Isaacs et al., 1986, 1990, 1994; Thormar et al., 1987; Orloff et al., 1993; Clarke and May, 2000). Exposure to 500 µg/ml monolaurin completely inhibited growth of cytomegalovirus in culture (Clarke and May, 2000).

Antimicrobial activity also was found for esters of more complex polyhydric alcohols (Conley and Kabara, 1973; Kato and Arima, 1971; Marshall and Bullerman, 1986a, b, 1994). Kato and Arima (1971) reported that the sucrose ester of lauric acid was active against a Gram-negative bacterium, whereas Conley and Kabara (1973) and Shibasaki and Kato (1979) indicated that these and other esters were primarily active against Gram-positive bacteria and fungi. In contrast to data generated for glycerides, the diester of sucrose, rather than the monoester, was more active. Sucrose dicaprylate possessed the highest activity of the sucrose esters but was still less active than monolaurin (Kato and Shibasaki, 1975a, b; Beuchat, 1980). The monoesters of glycerol and the diesters of sucrose not only have higher antimicrobial activity than their corresponding free fatty acids but also compare favorably in activity with commonly used antimicrobials, such as parabens, sorbic acid, and dehydroacetic acid (Tables 11.4 and 11.5). Sucrose palmitate combined with the bacteriocin nisin showed synergistic activity against Gram-positive bacteria but not Gram-negative bacteria (Thomas et al., 1998). Low levels of the combined compounds inhibited *L. monocytogenes*, *B. cereus*, *Lactobacillus plantarum*, and *S. aureus*. Sucrose laurate (1%) combined with high pressure (392 mPa) for 15 minutes at 45°C provided up to a 5-log reduction of *Bacillus subtilis* in milk, *B. cereus* in beef, *Bacillus coagulans* and *Alicyclobacillus* species in tomato juice, and *Alicyclobacillus* species in apple juice (Shearer et al., 2000). Enhanced inhibition of *E. coli* O157:H7 was achieved by 500 ppm sucrose monolaurate combined with 300 ppm EDTA compared to either compound used alone (Hathcox and Beuchat, 1996). Sucrose monolaurate enhanced thermal inactivation of the pathogen when used alone and was even greater when combined with EDTA. Beef slices that were contaminated with the pathogen and surface dipped in 1% to 3% sucrose laurate had significantly reduced populations compared with water dipped controls. Guava fruits atomized treated with 1% sucrose esters had less decay after 1 to 3 weeks of storage (Combrink et al., 1990). Others have shown similar effects. When 200 ppm sucrose esters were combined with 200 ppm tiabendazol, a *Penicillium* species citrus rot mold could be completely controlled on lemons (Bellone et al., 1993). A review of the antimicrobial properties of sucrose esters is available (Marshall and Bullerman, 1994).

Another series of food-grade esters was derived from a group of compounds called poly glycerol esters. These linear polymers of glycerol, discovered and advocated for food use by Babayan et al. (1964), were supplied to use for biocidal testing. As with sucrose esters, the medium-chain fatty acids appeared to be the most active (Conley and Kabara, 1973). It was of interest that, regardless of whether the polyol was tripolyglycerol, hexapolyglycerol, or decapolyglycerol, the fatty acid moiety seemed to determine overall biocidal activity. Generally, as the polyol became bulkier, the spectrum of biocidal activity became narrower. Other polyol esters have been tested, and except for special applications, none have proved to be more useful than monolaurin (Table 11.6).

GLYCEROL MONOLAURATE (MONOLAURIN)

Chronologically, one of the first reports of the commercial use of monolaurin as an emulsifier (under the trade name Lauricidin®) was to help solve a critical antifungal problem in diet margarine (Moustafa and Agin, 1980). Because the concentration of salt is lower and the amount of water is higher in diet margarine compared to regular margarine, the use of preservation levels of sorbate

TABLE 11.4
Comparison of Antifungal Activities of Fatty Acid Esters with Some Commonly Used Preservatives

Food additive	Minimum Inhibitory Concentration (vg/ml) ^a		
	<i>Aspergillus niger</i>	<i>Candida utilis</i>	<i>Saccharomyces cerevisiae</i>
Monocaprin	123	123	123
Monolaurin	137	69	137
Butyl-p-hydroxybenzoate	200	200	200
Sodium lauryl sulfate	100	400	100
Sorbic acid	1000	1000	1000
Dehydroacetic acid	100	200	200

^a By agar dilution method.

Source: Kato and Shibasaki (1975b).

TABLE 11.5
Comparison of Antibacterial Activities of Fatty Acid Esters with Some Commonly Used Preservatives

Food Additive	Minimum Inhibitory Concentration (µg/ml) ^a		
	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>
Sucrose dicaprylin	74	74	148
Monocaprin	123	123	123
Monolaurin	17	17	17
Butyl-p-hydroxybenzoate	400	200	200
Sodium lauryl sulfate	100	100	50
Sorbic acid	4000	4000	4000

^a By agar dilution method.

Source: Kato and Shibasaki (1975b).

TABLE 11.6
Minimum Inhibitory Concentrations of Various Polyglycerol Esters (mM) against *Corynebacterium* Species

Fatty Acid Moiety	Triglycerol	Hexaglycerol	Decaglycerol
Acetate	>3.55 ^a	>1.98 ^a	>1.25 ^a
Butyrate	>3.55 ^a	>1.98 ^a	>1.21 ^a
Caproate	2.96	>1.79 ^a	>1.17 ^a
Caprylate	1.37	0.85	1.13
Caprate	0.32	0.39	1.10
Laurate	0.24	0.38	0.53

^a No inhibition, maximum concentration tested.

Source: Conley and Kabara (1973).

was limited by taste and flavor considerations. The introduction of low levels of monolaurin helped produce an acceptable and marketable product. In the 30 years in which Lauricidin has been used, no product failure has resulted. In the single instance in which the monoglyceride was inadvertently omitted, fungal growth resulted.

Shibasaki (1982) stated, "Of all lipids evaluated lauric acid (esters) appear to have the greatest overall antimicrobial activity. Of these derivatives monolaurin would appear to have the highest potential for use in foods and cosmetics." This may be true for studies carried out in laboratory media, but monolaurin, as is true of other lipophilic preservatives, is influenced by the presence of starchy and proteinaceous material (Kato and Shibasaki, 1975b; Bal'a and Marshall, 1996b). To avoid this problem, the lower molecular-weight monoglyceride monocaprin was used in Japanese-style food. It is the opinion of the present authors that the use of monocaprin in foods is not warranted when monolaurin is properly used — that is, with homogeneous mixing (Cotton and Marshall, 1997); proper pH range (Bal'a and Marshall, 1996a); and effective acidulants such as acetic, citric, or lactic acids (Oh and Marshall, 1994). The purity of the monoglyceride is very important. Many monoglyceride preparations have a number of impurities, particularly notable quantities of di and tri esters. It was found that monoglycerides less than 90% monoester were not effective antimicrobials, which is why Kabara coined the name Lauricidin® to emphasize its high monoester content.

An effective teat dip preparation using Lauricidin, saturated fatty acids, and lactic acid has been described (Boddie and Nickerson, 1992). The treatment reduced mammary infections by *S. aureus* and *Streptococcus agalactiae* 40% to 80%. Other possible applications of monolaurin in foods have been explored by Kato and Shibasaki (1975c), who demonstrated that lauric acid, monocaprin, and monolaurin significantly enhanced the thermal death of *E. coli* and *Pseudomonas aeruginosa*. The thermodynamic characteristics of bactericidal action of monolaurin and heat were studied by Tsuchido et al. (1981), who found that the bactericidal effect of increasing concentrations of monolaurin would be more effective in low-temperature, long-time treatments than high-temperature, short-time processes.

The usefulness of monolaurin in the heat inactivation of spores was substantiated by Kimsey et al. (1981), who showed enhanced thermal inactivation of *Bacillus stearothermophilus* 1518 in the presence of 18 mM monolaurin. The decimal reduction time (D value, the time required to reduce the number of viable spores by 90%) at 120°C was reduced by 49.8%, whereas the D value for *B. subtilis* was reduced by 47.9%. The effectiveness of various saturated monoglycerides on *B. stearothermophilus* spores is shown in Table 11.7. Monocaprin and monolaurin were the most active of the series C8 to C16. Monolaurin was also tested in 10% nonfat dry milk and in the fluid portion of cream-style corn. In these foods the $D_{120^{\circ}\text{C}}$ time was reduced by approximately 50%. It was concluded that monolaurin could be used as a food additive (emulsifier) to reduce the heat treatment required to achieve commercial sterility of foods. Naruki et al. (1983) confirmed earlier studies by demonstrating that fatty acids and monoglycerides inhibited *B. subtilis*. Fatty acids were less active than their corresponding monoglyceride. What was not explained was the loss of heat resistance of spores during germination by the addition of lipid additives (Table 11.8).

Monolaurin or lauric acid, both at 0.5%, were able to eliminate *B. subtilis* from a water-in-oil emulsion stored at 23°C (Plockova et al., 1999b). The same treatments also reduced the growth of *E. coli* in the emulsion. A combined treatment of 0.005% monolaurin and 1.5% lactic acid was able to inactivate (>8 log reduction) *E. coli* O157:H7 within 15 minutes at 40°C (Venkitanarayanan et al., 1999). A 5-log reduction was observed at 22°C using the same combination. A treatment combination of alkaline shock (pH 10.5), high salt stress (10% NaCl), and 100 ppm monolaurin or lauric acid was able to achieve a 5- to 8-log reduction in populations of *L. monocytogenes*, *Pseudomonas fragi*, and *P. fluorescens* (Vasseur et al., 2001).

For dairy applications, a 35% increase in cottage cheese shelf life (5 to 10 days longer) can be obtained using 500 ppm monolaurin (Bautista et al., 1993). The shelf life increase was accomplished as a result of a 100-fold reduction in the number of spoilage bacteria in the

TABLE 11.7
Effectiveness of Various Saturated Monoglycerides on *Bacillus stearothermophilus* Spores at 120°C

Spores Heated in 18 mM	Number of C in Chain	Average D ₁₂₀ (s)	Range
Control	—	265	236–294
Monocaprylin	8	195	180–210
Monocaprin	10	88	84–91
Monolaurin	12	119	117–120
Monomyristin	14	221	210–228
Monopalmitin	16	244	234–248

Source: Kimsey et al. (1981).

TABLE 11.8
Effect of Dodecanoic Acid and Its Monoglyceride on the Loss of Heat Resistance of *Bacillus subtilis* Spores during Germination^a

Exposure Time (min)	Viability (%) after Heat Treatment of Spores		
	None	DA ^b (0.2 mM)	GMD ^c (0.05 mM)
0	100	100	100
30	11.5	59	55
60	6.4	35	33

^a Spores exposed to fatty acid, then heat treated at 85°C for 30 minutes; viability expressed as percentage of initial spore count (approximately 10⁷ CFU/ml).

^b DA, dodecanoic acid.

^c GMD, glycerol monododecanoate.

Source: Nakuri et al. (1983).

monolaurin-containing product. Unfortunately, sensory results demonstrated that trained panelists could detect 250 ppm monolaurin in cottage cheese as a notable chemical off-flavor, which would limit its use in this application. Others (Bell and DeLacy, 1987) have noted a soapy odor in luncheon meat treated with 500-ppm monolaurin. Kappa-conjugated linoleic acid and monolaurin were found to be effective killing agents of *L. monocytogenes* in broth (Wang and Johnson, 1992). Monolaurin was effective to inactivate *L. monocytogenes* in skim milk but not whole milk, presumably because of higher fat content in the latter food. K-conjugated linoleic acid was an effective bacteriostat in preventing growth of *L. monocytogenes* in whole and skim milk at 4°C. A combined treatment of 250 ppm monolaurin combined with 100 IU/ml nisin was able to eliminate 7-log populations of *B. cereus*, *B. coagulans*, *B. subtilis*, and *Bacillus licheniformis* in milk (Mansour et al., 1999; Mansour and Milliere, 2001). Italian Stracchino cheese surface treated with 200 µg monolaurin/cm prevented growth of *L. monocytogenes* during storage at 5°C for 12 days (Stecchini et al., 1996).

Clostridium sporogenes and *L. monocytogenes* inactivation by cooking was studied by Unda et al. (1991). Beef roasts were pumped with brine containing phosphates and acetic acid, sodium lactate, potassium sorbate, or monolaurin. The meat was cooked in the bag once or twice to 62.8°C. In roasts recooked after being inoculated externally, monolaurin and sodium lactate were very effective in inhibiting anaerobic bacteria during temperature abuse. These results suggest that perhaps combinations of monolaurin and sodium lactate may offer potential control of clostridia and listeriae

TABLE 11.9
Bacterical Effect of Fatty Acids and Their Esters against
Escherichia coli

Chemical Agent	Surviving Fraction after Frozen Storage ^a
Nonionic surfactants (100 ppm)	
None	1×10^{-1}
Tween 20	1×10^{-3}
Tween 40	3×10^{-2}
Tween 60	2×10^{-2}
Tween 80	2×10^{-2}
Span 20	1×10^{-6}
Span 60	4×10^{-2}
Span 80	2×10^{-2}
Span 85	1×10^{-2}
Monoglycerides (10 ppm)	
Monocaprylin	3×10^{-2}
Monocaprin	1×10^{-4}
Monolaurin	1×10^{-6}
Monomyristin	5×10^{-5}
Monopalmitin	2×10^{-4}
Monostearin	3×10^{-2}
Monoolein	1×10^{-4}

^a Surviving fraction of every unfrozen control was $>10^{-1}$.

Source: Takano et al. (1979).

in “microwave-ready” beef products. In their paper, Unda et al. (1991) confirm the instability of refrigerated, precooked, nitrite-free, microwave-ready meat products as prepared at present. These observations should be viewed with concern by the meat industry and regulatory agencies.

In addition to enhancement of thermal death by monolaurin, Takano et al. (1979) showed freezing significantly injured cells of Gram-negative bacteria exposed to monolaurin. Of the various nonionic derivatives tested, monolaurin was the most active in reducing viability (Table 11.9). Carbohydrate fatty acid esters enhance damage in the growth phase rather than the freezing process. Freezing with these agents was also found to be lethal to Gram-positive bacteria.

Although Ueda and Tokunaga (1966) and Koga and Watanabe (1968) reported on the action of monocaprylin and monocaprin in soy sauce, Kato (1981) made a direct comparison of fatty acids, monoglycerides, and sucrose fatty acid esters. He found that capric acid and monolaurin were the most active preservatives in soy sauce that contained osmophilic yeast. In a series of sucrose esters, sucrose monolaurate was more active than sucrose monocaprate. The activity of monolaurin compares with that of sodium lauryl sulfate and was superior to butyl-p-hydroxybenzoate, which was approved as an antimicrobial additive for soy sauce. Shibasaki et al. (1979) added several different glycerol and sucrose esters to tofu (soybean protein curd) and placed the mixture in a casing kept at 30°C for 5 days. Coliform, total, and thermostable bacterial counts were less than 300 per gram for the product. Controls produced by conventional methods showed levels of 8.3×10^3 , 2.0×10^5 , and 3.5×10^5 colony-forming units per gram after storage, respectively.

Smith and Palumbo (1980) investigated the inhibition of aerobic and anaerobic growth of *S. aureus* in a model sausage system. They compared potassium sorbate, sorbic acid, and monolaurin. Whereas anaerobic growth of *S. aureus* was inhibited by 2500 ppm sorbate and monolaurin, twice as much was required to inhibit aerobic growth. To enhance activity it was necessary to acidify the

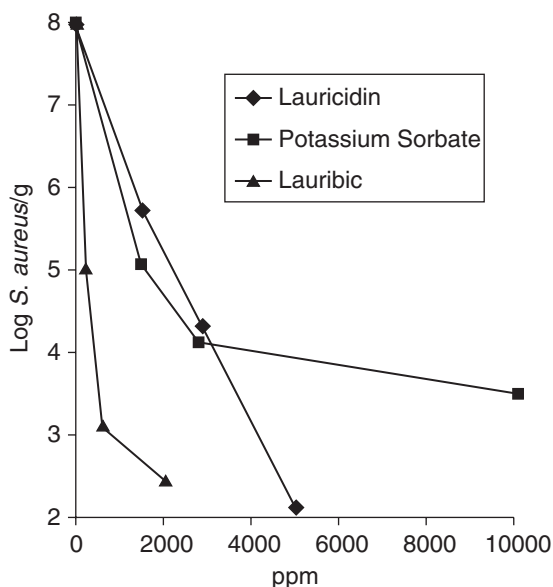


FIGURE 11.1 Effect of Lauricidin®, potassium sorbate, or Lauribic™ on survival of *Staphylococcus aureus* in a model sausage after 3 days at 35°C (From Kabara, 1984).

system with 20 mg of lactic acid. Under these same acidulating conditions, sorbic acid and Lauribic™ (a mixture of sorbic acid and monolaurin produced by Med-Chem Labs) inhibited both aerobic and anaerobic growth equally well at 500 and 750 ppm, respectively. In these experiments, the amount of lactic acid added, not the pH, was critical. The addition of 10 to 20 mEq lactic acid resulted in a small change in pH value (0.4 units) and no change in microbial titer. The addition of 25 and 30 mEq acids resulted in a pH decline of 0.6 units to pH 5.7 but a decrease in the preserved meat emulsion of 4 to 5 logs from control levels. Thus, when sorbic acid is added to such a test system, one is adding not only a preservative but also an acidulating agent. When comparisons between sorbic acid and monolaurin are made, this important factor should be recognized.

Using the same model sausage system and controlling pH, Kabara (1984) showed that monolaurin was slightly more active than potassium sorbate. Lauribic™ gave greater inhibition than either monolaurin or sorbic acid alone. Inhibition was greater against anaerobic than aerobic growth. In the latter instance, high (>10,000 ppm) concentrations of sorbate and lower (5000 ppm) amounts of monolaurin were required for a good reduction in the bacterial population. Lauribic™ was effective at 500 ppm (Figure 11.1).

A Japanese patent (Katagiri et al., 1980) disclosed a preservative system for meat, fish, vegetables, and processed foods formulated with combinations of monolaurin, phosphates, and sorbates. First, 1 part monolaurin, 20 parts of a mixture of pyrophosphate and polyphosphate (1:1), and 40 parts sorbic acid coated with hydrogenated soybean oil were mixed. The preservative mixture was added to sausage so that the final concentration of sorbic acid was 0.3%. The product stored at 25°C showed no spoilage before 11 days, whereas controls spoiled in 3 days.

Robach et al. (1981) made direct comparisons between monolaurin and sorbic acid in laboratory media, cottage cheese, and a pork homogenate. In general they found sorbic acid more active than monolaurin, but they reported that monolaurin under similar conditions was more effective than sorbic acid against the growth of *Salmonella Typhimurium*. Contrary to the results of others, Robach et al. (1981) found no advantage to use of monolaurin alone or monolaurin-sorbic acid combinations in their systems. Other studies suggested that combinations of monoglycerides and fatty acids were

useful. Yuda et al. (1977) reported that organic acids, such as fumaric acid, when combined with lauric esters of propylene glycol, glycerol, and polysorbate 80 (sorbitan) helped preserve starch-containing foods. Monolaurin and fumaric acid added to kamaboko (fish paste) at levels of 0.12% and 0.05% were effective preservatives. In addition to fumaric and sorbic acids, a wide-spectrum food preservative system used lactic acid as the acidulant (Katagiri and Yamamoto, 1978). These investigators designed a combination that included a 0.5% monocaprin–0.2% monolaurin mixture, lactic acid, glycine, and calcium lactate. This mix was added to kamaboko at 0.8% and preserved the product for 20 days at 30°C. The control product spoiled in 6 to 10 days. Ogawa (1980) used one or more salts of acetic, citric, tartaric, malic, and lactic acids to potentiate the effect of saturated fatty acid (C8 to C12) monoglycerides. Their use improved the flavor and aroma of frozen cooked foods. Oh and Marshall (1992, 1993a) demonstrated that low pH improved monolaurin efficacy against *L. monocytogenes*.

The utility of monolaurin in meat products is underscored by data generated by Notermans and Dufrenne (1981). Monolaurin (Lauricidin®), when used in the proportion of 5 g/kg meat slurry (pH 6.0 to 6.2), completely inhibited toxin production by *C. botulinum* types A, B, and E. The inhibitory effect of monolaurin was not improved by the addition of lactic acid to the meat slurry until the pH was lowered to less than 5.2. Under these same conditions, potassium sorbate inhibited toxin production of type B at 32 g/kg (32,000 ppm, or 3.2%). Because monolaurin was 6 times more active than potassium sorbate, Notermans and Dufrenne (1981) recommended it as a possible alternative to nitrite for the preservation of meat products. Because nitrite is necessary for the color and taste of meat products, the suggestion to reduce rather than eliminate nitrite seems to be indicated. High concentrations of monolaurin (2.5%) were shown to inhibit *C. sporogenes* and *L. monocytogenes* in cooked beef roasts (Stillmunkes et al., 1993). Monolaurin activity was less than sodium lactate, particularly in roasts that were temperature abused. In addition, monolaurin caused significant purge during storage of the roasts, which is a quality defect.

A wider application of monolaurin in meat products was reported by Baker et al. (1982). They added Lauricidin® to deboned chicken meat, minced fish, and chicken sausage. Spoilage for these meat systems was evident in 5, 8, and 9 days, respectively. The addition of 0.2% citric acid and 250 ppm Lauricidin® increased the shelf life of deboned chicken meat by 7 days, minced fish by 5 days, and chicken sausage by 8 days. Hall and Maurer (1986) tested Lauricidin® and propylene glycol as inhibitors of *C. botulinum* in turkey frankfurter slurries as possible replacements for nitrite as a preservative, or so that lower nitrite levels could be used. They reported that inhibition of *C. botulinum* was achieved; however, it was not clear from the report what monolaurin concentration was used.

The action of Lauri-lac™ (a mixture of monolaurin and lactic acid) on the multiplication of *Salmonella* Typhimurium in minced beef was examined at 0°C to 2°C, 30°C, and 37°C (Catsaras et al., 1987). The effects at lower temperature and concentrations between 0.1% and 0.5% were negligible. At 37°C and 0.5%, the original inoculum was reduced from 2.5×10^5 CFU/g to less than 10^2 in the time period examined. This confirms similar studies that suggest that monoglyceride preparations are more effective under abusive temperature conditions. As reported by Duxbury (1986), Kabara and Brady (Michigan State University) found that control cheese samples and those containing 0.05% or 0.10% monolaurin had a shelf life of only 5 to 8 days. Lactic acid (0.2%) alone extended the shelf life to 25 days, but the addition of 0.2% monolaurin plus lactic acid increased the time to 120 days. Preparation of the cheese product at 25°C and 80°C demonstrated that the pasteurization of a heat-sensitive product could be achieved at lower temperatures. This combination of monolaurin and acidulant was also successfully used in an onion–cucumber powdered snack dip.

Nisin, sorbic acid, Lauricidin®, and Lauribic™ were studied by Bell and DeLacy (1987). Determinations were made of their ability to prevent *B. licheniformis* growth in a model cooked luncheon meat product. This product was made from a 3:1 mixture of lean beef and beef fat and contained 2.5% salt, 0.3% sodium tripolyphosphate, and 200 ppm sodium nitrite. Various

concentrations of the test preservatives were added to batches of the raw meat emulsion inoculated with 2×10^4 *B. licheniformis* spores per gram. Emulsions were cooked to a pasteurization value of 36 (reference temperature 70°C), chilled overnight, and then stored at 25°C. Minimum concentrations of the test preservatives necessary to prevent product spoilage within 10 days during abusive storage were 500 IU nisin/g, 0.125% sorbic acid, 0.5% Lauricidin®, and 0.25% Lauribic™. These concentrations also were effective in controlling the growth of natural *Enterococcus* species spoilage microflora in mildly cooked products. The minimum effective concentration (0.125%) of sorbic acid was made more effective by monolaurin. A slow increase in enterococci growth in the presence of monolaurin indicated a persistent effect, whereas a rapid increase observed with both nisin and sorbate reflected their decay in the stored product. Thus, combinations of sorbic acid and monolaurin may provide additional protection beyond that produced by a given concentration of sorbic acid used alone.

The bacteriocin nisin was found to be more effective in the presence of monolaurin (Blackburn et al., 1989). Nisin at concentrations up to 2000 ppm was not active in complex milk medium. Monolaurin also was not active; however, a combination of the two agents was extremely potent against *S. agalactiae*. The combination was more than 100 times more active than what would be expected for an additive effect and 10,000 times more active than either of the components individually.

A treatment combination of modified atmosphere packaging, monolaurin, and lactic acid was able to reduce growth of *L. monocytogenes* in crawfish tail meat (Oh and Marshall, 1995a). Similarly, monolaurin combined with lactic acid was able to reduce the population of *L. monocytogenes* on catfish fillets (Verhaegh et al., 1996). Preharvest and postharvest control of the emerging seafood-borne pathogen *Streptococcus iniae* may be achieved using a combination of low to moderate pH, salt, and monolaurin (Trotter and Marshall, 2003). Biofilms of *L. monocytogenes* on stainless steel were eliminated using combinations of monolaurin with moderate heat (Oh and Marshall, 1995b) or acetic acid (Oh and Marshall, 1996).

Chipley et al. (1981) was interested in the antifungal and antiaflatoxin effects of monolaurin. In their study, monolaurin, Lauribic™, and sorbic acid were tested for aflatoxin inhibition using aflatoxigenic molds *Aspergillus flavus* and *Aspergillus parasiticus*. Sorbic acid levels of 1000 ppm were required for inhibition of aflatoxin formation, but monolaurin and Lauribic™ were active at 750 ppm. Mycelial growth inhibition also was significant, with controls reaching an average of 952 mg mycelia per flask; sorbic acid, Lauricidin®, and Lauribic™ treatments only reached 540, 582, and 480 mg mycelia per flask, respectively. Because the molecular weight of sorbic acid is 112.1 and that of Lauricidin® is 274.4, Lauricidin® is approximately 2.4 times more active on a mole per mole basis. Mansour et al. (1996) found that monolaurin was a less effective inhibitor of nontoxicogenic aspergilli and penicillia than were eugenol or isoeugenol. Monolaurin (100 to 200 ppm) was still capable of significantly reducing mycelial dry weight and aflatoxin B1 and G1 in growth media. Lisker and Paster (1982) carried out extensive studies with Lauricidin® and related food-grade agents as potential antifungal agents for citrus products. Three different commercial sources of monolaurin were tested against 16 fungi belonging to different groups having different cell-wall compositions. The results are presented in Tables 11.10 and 11.11. Lauricidin® 812, 802, and 112 showed higher antifungal activity than Lauricidin®. Lauricidin® 812 was more active against fungi, whereas Lauricidin® 112 was very active against yeasts. The combination of Lauricidin® and sorbic acid (Lauribic™) proved to be more active than either compound alone. The most effective combination was Lauricidin® Plus F, a mixture of monolaurin, tert-butylhydroxyanisole, and EDTA. Lauric acid and its glycerol esters were tested against several food spoilage molds of the genera *Alternaria*, *Cladosporium*, *Fusarium*, *Geotrichum*, and *Penicillium*. The order of effectiveness in decreasing order was monolaurin, dilaurin, trilaurin, and lauric acid (Plockova et al., 1999a).

Digestion of pectin to molecular weights of 600 to 5000 produces antimicrobial agents (Koki et al., 1989). With a combination of such pectin digestions, antifungal activity can be enhanced by

TABLE 11.10
Antifungal Activity of Lauricidin and Derivatives^a Compared with Sorbic and Propionic Acids^b

Fungi	Lauricidin plus F ^c	Lauricidin	Sorbic Acid	Lauribic ^d	Propionic Acid
<i>Pythium elongatum</i>	100	10.0	100	100	100
<i>Phytophthora citrophthora</i>	100	41.2	100	100	100
<i>Mucor circinelloides</i>	100	30.5	94.3	100	78.7
<i>Rhizopus stolonifer</i>	100	10.1	100	100	100
<i>Aspergillus flavus</i>	100	28.6	83.5	100	79.9
<i>Aspergillus ochraceus</i>	100	21.1	73.5	100	84.1
<i>Aspergillus niger</i>	100	40.3	100	100	97.6
<i>Penicillium digitatum</i>	100	39.3	100	100	100
<i>Penicillium patulum</i>	100	18.9	75.2	100	65.7
<i>Fusarium graminearum</i>	100	20.1	100	100	100
<i>Saccharomyces cerevisiae</i>	100	26.5	100	100	23.2
<i>Candida albicans</i>	100	17.5	100	100	11.7
<i>Rhodotorula mucilaginosa</i>	100	98.0	100	100	100
<i>Sporobolomyces</i> sp.	100	100.0	100	100	100
<i>Sclerotium rolfsii</i>	100	63.3	100	100	100
<i>Rhizoctonia solani</i>	100	54.9	98.2	100	100

^a At a concentration of 1%.

^b Results are expressed as the percentage of mycelial growth inhibition.

^c Mixture of monolaurin, tert-butyl hydroxyanisole, and ethylenediamine tetraacetic acid.

^d Combination of monolaurin and sorbic acid.

Source: Lisker and Paster (1982).

TABLE 11.11
Antifungal Activity of Lauricidin Plus F, Lauribic^a, and Lauricidin Derivatives^{b,c}

Fungi	Lauricidin plus F ^d	Lauribic ^e	Lauricidin	Lauricidin 812	Lauricidin 802	Lauricidin 112
<i>Pythium elongatum</i>	100	100	32.6	100	100	88.0
<i>Phytophthora citrophthora</i>	100	100	70.5	100	100	89.1
<i>Mucor circinelloides</i>	100	48.5	56.4	55.3	64.2	82.7
<i>Rhizopus stolonifer</i>	100	81.0	29.8	42.7	69.9	79.4
<i>Aspergillus flavus</i>	85.5	44.8	73.6	75.0	65.8	64.5
<i>Aspergillus ochraceus</i>	90.5	40.4	53.4	100	100	70.3
<i>Aspergillus niger</i>	89.4	48.3	71.7	100	89.6	82.7
<i>Penicillium digitatum</i>	86.7	100	76.1	100	100	100
<i>Penicillium patulum</i>	85.1	24.6	34.3	100	38.3	40.1
<i>Fusarium graminearum</i>	82.5	100	57.5	100	100	66.1
<i>Saccharomyces cerevisiae</i>	100	100	56.5 ^a	51.7 ^a	92.7 ^a	100 ^a
<i>Candida albicans</i>	0	100	0 ^a	0 ^a	35.5 ^a	100 ^a
<i>Rhodotorula mucilaginosa</i>	95.7	100	9.2 ^a	50.0 ^a	100 ^a	100 ^a
<i>Sporobolomyces</i> sp.	100	100	79.9 ^a	91.2 ^a	90.4 ^a	100 ^a
<i>Sclerotium rolfsii</i>	100	100	65.6	93.4	98.7	99.1
<i>Rhizoctonia solani</i>	100	100	61.7	85.8	89.7	89.3

^a At a concentration of 0.05%.

^b At a concentration of 0.5%.

^c Results are expressed as the percentage of mycelial growth inhibition.

^d Mixture of monolaurin, tert-butyl hydroxyanisole, and ethylenediamine tetraacetic acid.

^e Combination of monolaurin and sorbic acid.

Source: Lisker and Paster (1982).

adding at least one member of glycerol fatty acid esters and disodium EDTA. The glycerol esters mentioned were esters of a saturated fatty acid having 8 to 12 carbon atoms or unsaturated fatty acids having 18 carbon atoms.

The bactericidal activity of ethanol can be enhanced in the presence of certain food additives (Furuta et al., 1984). Of the substances studied, monoglycerides (monocaprin) showed strong enhancing effects. The effects appeared to be synergistic. Oh and Marshall (1993b) found similar enhanced activity of ethanol when combined with monolaurin against *L. monocytogenes*. Blaszyk and Holley (1998) found enhanced activity of monolaurin when combined with either eugenol (a volatile phenolic compound) or sodium citrate against a number of meat spoilage and pathogenic bacteria. Lactic acid bacteria and *E. coli* O157:H7 were most resistant, and *L. monocytogenes* and *B. thermosphacta* were most sensitive to control.

Saturated monoglycerides (C6 to C12) inhibit mutagenesis by N-nitroso-dimethylamine in cultures of *E. coli* (Japanese Patent, 1985). Thus, a food additive consisting of monoglyceride (C8 to C12), glycerin, and gelatin inhibited nitrosamine mutagenesis. This finding may be of importance to cured meat processors desiring to control nitrosamine formation in their products.

MECHANISM OF ACTION

Some specific examples are provided to build a working hypothesis for understanding the mechanism of fatty acids and their esters as antimicrobial agents. Unlike antibiotics, fatty acids and their esters have several modes of action that are nonspecific. Thus, development of resistance to these compounds has not been reported. Bactericidal concentrations of long-chain fatty acids were shown to stimulate oxygen uptake of *Bacillus megaterium* and *Micrococcus lysodeikticus* at pH 7.4. These concentrations approximated those producing protoplast lysis with fatty acid chain lengths greater than capric acid. Higher concentrations of the fatty acids produced complete inhibition of oxygen uptake. The order of activity of the fatty acids was similar to that published for other antibacterial effects (Galbraith et al., 1971; Galbraith and Miller, 1973a, b). The greater effect of the *cis* isomer of oleic acid compared to the *trans* isomer, elaidic acid, was clearly demonstrated. Protoplasts and whole cells of *M. lysodeikticus* were more resistant to inhibition by the fatty acids than those of *B. megaterium*, and this reflects the relative behavior of the two bacteria in the bactericidal and lytic solution. Similarly, the limited response of the oxygen uptake mechanisms of *Pseudomonas phaseolicola* and *E. coli* reflected the resistance of these bacteria to the bactericidal activity of fatty acids. Partial removal of the cell wall, however, resulted in a marked sensitivity to inhibition of oxygen uptake, and this strongly suggested that cell-wall lipids may be protecting the cytoplasmic membrane from the fatty acids following absorption by the cells (Galbraith and Miller, 1973a). Hamilton (1970) also observed that the formation of spheroplasts from Gram-negative bacteria rendered them more susceptible to metabolic inhibition by lipid-soluble antibacterial agents. These effects on the aerobic respiratory activity of bacteria provide evidence that the membrane is a site of action of fatty acids, especially because enzymes involved in oxygen uptake are membrane bound (Storek and Wachsmann, 1957).

In contrast to the irreversible effects associated with the inhibition of respiratory activity, the inhibition of the membrane-located transport of amino acids into cells of *B. megaterium* and *M. lysodeikticus* was observed at levels that reversibly inhibited the uptake (Galbraith and Miller, 1973c). Lower concentrations of fatty acids stimulated uptake of amino acids into the cells. Others also have observed this effect, as well as the marked inhibition of amino acid uptake in the presence of higher concentrations of oleic and linoleic acids (Gale and Folkes, 1967; Gale and Llewellyn, 1971). The bacteriostatic nature of the inhibition of amino acid uptake in addition to the sublethal concentrations used was evident. The order of activity of the fatty acids was similar to that reported previously for bactericidal effects, protoplast lysis, and inhibition of oxygen uptake. Unsaturated fatty acids were more effective than the most effective saturated fatty acids, lauric acids, and myristic

acids. The inhibition of amino acid uptake into cells of *M. lysodeikticus* again required higher concentrations than were necessary to similarly affect *B. megaterium* (Galbraith and Miller, 1973c).

In general, uptake of glutamic acid by *S. aureus* was more sensitive to inhibition than that of lysine (Hamilton, 1968). Greater sensitivity of glutamic acid uptake to inhibition by tetrachlorosalicylanilide (TCS), a membrane-perturbing compound, compared to that of lysine was the result of the total dependence of glutamic acid alone on energy for transport. The uptake of lysine by bacteria also may be incompletely energy dependent and hence less sensitive to inhibition owing to the postulated energy-inhibiting action of the fatty acids. It is of interest that fatty acids markedly inhibited the uptake of lysine and glutamic acid by *C. perfringens*. This inhibition was more effective than either dinitrophenol (DNP) or sodium azide. Harold and Baarda (1968) observed that uncouplers of oxidative phosphorylation inhibited the energy-dependent transport of amino acids anaerobically with glycolyzing cells of *E. faecalis*. These authors proposed that these compounds interfered with utilization of the energy required for membrane transport because the generation of cellular adenosine triphosphate (ATP) and its participation in biosynthesis were not affected.

Fatty acids at sublethal levels also induced the leakage of C^{14} -labeled amino acids from preloaded *B. megaterium* cells. Sodium azide and DNP also promoted leakage of amino acids, and it is probably that the release from the amino acid pool occurred in response to the inhibition of the energy-uncoupling systems necessary to maintain their presence in the pool, as observed by Hamilton (1968) with TCS. As indicated previously (Galbraith and Miller, 1973b), fatty acids did not appear to induce nonspecific leakage from the cells. Sheu et al. (1972) showed that in both whole cells and isolated membrane vesicles, short-chain fatty acids noncompetitively inhibit the uptake of amino acids. The amino acid transport system was energized by the cytochrome-linked electron transport system (Kaback and Milner, 1970; Konings and Freese, 1971). In contrast, the uptake of α -methyl glucoside or fructose, which is affected by the phosphoenolpyruvate transferase system, was only minutely inhibited (Kaback, 1970; Kundig and Roseman, 1971). Long-chain fatty acids seem to have the same mechanisms of action (Sheu and Freese, 1973).

It is evident that the mode of action of and the type of inhibition produced by the fatty acids depend on the concentration used. It also is apparent that at high concentrations the effects are irreversible and bactericidal. Under these conditions (at pH 7.4), the fatty acids in general induced lysis of protoplasts and stimulated uptake of oxygen, and in this respect their activity resembled that found with mitochondria. Scholefield (1963) reviewed the effects found with mammalian mitochondria, and these include stimulation and, at higher concentrations, inhibition of oxygen uptake, uncoupling of oxidative phosphorylation, and interference with inorganic phosphate transfer from ATP. As with bacteria, lauric and myristic acids were the most active saturated fatty acids, but they were less effective than the C18 unsaturated acids. It is probable that the stimulation of oxygen uptake at low bactericidal concentrations was induced by the uncoupling of oxidative phosphorylation that produced disaggregation of the membrane. That normal respiratory activity is dependent on the maintenance of membrane integrity may be deduced from the fact that stimulation of oxygen uptake occurs at the concentrations that produce lysis of protoplasts. Similarly, with mitochondria, Green and Perdue (1966) emphasized the importance of the steric configuration in the functioning of the energy system, and Scholefield (1963) reported that the uncoupling activity of venom lecithinase was the result of the disruption of the lipids necessary to preserve mitochondrial structure. The fatty acids could exert their bactericidal activity by producing irreversible distortions of bonding as a result of surfactant effects on the bacterial cell membrane, which could dislocate the components of the energy system and inhibit the synthesis of ATP. Evidence for this was provided by Hardesty and Mitchell (1963), who showed that fatty acids interact with cytochrome C in the mitochondrial energy. Similar evidence was found for bacteria with Fenticlor, which inhibited amino acid transport in bacterial cells as a result of interference with the coupling of energy to transport (Hamilton, 1968; Hugo and Bloomfield, 1971). These workers, however, observed that these antibacterial agents produced both stimulation of oxygen uptake and inhibition of energy-dependent amino acid transport at bacteriostatic concentrations.

The fatty acids specifically exert their activity by uncoupling energy systems, and it appears that the fatty acids have many properties in common with the temperature-active antibacterial agents such as tetrachloroosalicylanilide (Hamilton, 1970), trichlorophenol (Wolf and Schaeffer, 1968), and Fenticlor (Hugo and Bloomfield, 1971). Another mechanism of action can be deduced from the fact that fatty acids are known to decrease glycolysis and stimulate gluconeogenesis (Sheu et al., 1975). Growth inhibition of *B. subtilis* by fatty acids was reduced by glycolytic compounds, especially glucose and fructose, but only slightly or not at all by compounds in the citric acid cycle (Coutelle and Schewe, 1970). Growth inhibition by fatty acids was less pronounced when the cells were grown in a synthetic medium and grown more slowly. Presumably the intracellular production or uptake was inhibited and became more easily growth rate limiting in rich rather than in minimal media.

Because membranes of all organisms and cells could be affected by lipophilic substances, a comparison of the growth, transport, and nerve function of human cells with those of microorganisms may reveal preservatives that are least harmful to humans (Coutelle and Schewe, 1970). The growth inhibition of bacteria has been attributed to the inhibitors of amino and keto acid transport (Coutelle and Schewe, 1970), whereas that of human cells has remained unexplained. The labors of Freese and collaborators shed some light on this problem (Sheu et al., 1975). In human fibroblasts of HeLa cells, neither amino acid uptake nor ATP synthesis was inhibited by fatty acids at concentrations that completely inhibited growth. Therefore, lipophilic drugs have different modes of action on bacterial and mammalian cells. This difference may account for their lack of toxicity in mammals.

The mechanism emphasis on lipophilic fatty acids, rather than their esters, is based primarily on a lack of investigation of the latter. The action of fatty acids and monoglycerides on an NADH₂ oxidase system was similar (Coutelle and Schewe, 1970). Activity of the system was depressed by 50% using 0.64 nmol fatty acids or 0.14 nmol monoglycerides per gram protein. The effect of these two lipid classes on cellular respiration also was studied. The results of these investigations, together with those testing the inhibitory effects of the monoglycerides on various enzyme systems, showed that monoglycerides act only on the oxygen side of the flavin in NADH₂ dehydrogenase. The fatty acids, however, were less specific inhibitors acting on several sites. Monolaurin is known to produce highly ordered membranes, which may interrupt membrane function (Taylor et al., 1975). Ved et al. (1990) demonstrated that monolaurin acted synergistically with penicillin G at concentrations below its minimum micelle concentration. This finding suggests that the mechanism of monolaurin inhibition of bacteria is not because of its detergent activity, which requires micelle formation. This finding supports the notion that monolaurin activity against bacteria is not the result of physical stresses on cell membranes.

Staphylococcus aureus production of lactamase, toxic shock toxin 1, and other exoproteins can be inhibited by monolaurin (Schlievert et al., 1992; Projan et al., 1994). In addition, monolaurin blocks the induction of vancomycin resistance by *E. faecalis* (Ruzin and Novick, 1998). These inhibitory effects by monolaurin were presumed to be the result of interference with membrane-associated signal transduction resulting in transcriptional blockage of promoters (Ruzin and Novick, 2000).

FUTURE APPLICATIONS

The ever-dwindling list of chemicals useful to preserve foods has raised a challenge to the food technologist. We believe the solution is not the discovery of new chemicals with preservation potential but, rather, imaginative application of food additives currently approved. This is a possibility because it has been discovered (Jindal and Marshall, 1996) that monoglycerides, food-grade phenolics, and chelating agents all have biocidal properties, individually as well as collectively. The key idea is to use these food-grade chemicals in a systems approach (multiple barrier or hurdle technology) to food preservation. A systems approach to preservation maximizes the multifunctional

properties of chemicals so that fewer and fewer chemicals can satisfy more needs of the food processor. For example, monoglycerides have been used as emulsifiers in the food industry for more than 80 years. The technology developed for this class of compounds and their record of safety (generally recognized as safe, GRAS, status) allows us to apply monoglycerides to foods with great confidence. The same arguments can be raised for the phenolic antimicrobials methyl and propyl paraben and, more specifically, tert-butyl hydroxyanisole (BHA), which is both a phenol and an antioxidant (Brannen et al., 1980). Food-grade chelators such as EDTA, citric acid, and lactic acid are additional chemicals that can potentiate the effect of fatty acid-based preservatives and act as deterrents to the chemical oxidation of foods.

Preservation combinations using monolaurin, a food-grade phenolic and chelator, have been formulated (Kabara, 1978, 1979b, 1980). Such combinations are more effective than the individual chemicals alone. These combinations have been primarily applied to the cosmetic and pharmaceutical industry, but their potential application to the food industry is obvious and perhaps underexploited by the food industry.

As in any systems approach, the number and level of variables used depend on the problem to be solved. Along with determining the level of material to be used, their ratio to each other is somewhat important. For many applications a ratio of 1:1:1 for three food additives (monoglyceride-food-grade phenolic-chelator) has been found satisfactory (Kabara, 1978, 1979b, 1980). Although the amount of phenol (1000 ppm) and EDTA (100 ppm) is limited for food use, the amount of monolaurin that can be added is restricted more by taste than by governmental regulation. For most applications, 500 to 750 ppm of monolaurin is effective and remains undetected by most sensory panelists (Kabara, unpublished data). Higher application levels need not be discouraged, particularly when special problems exist and the bitter or soapy taste can be masked. An obvious example where higher concentrations may be valuable is in carcass and produce wash applications.

Kato and Shibasaki (1976) described the usefulness of acidulants in increasing the antimicrobial spectrum and activity of monoglycerides, particularly monolaurin. Chemical agents were selected from the standpoint of safety. Citric, lactic, malic, and polyphosphoric acids were found to enhance the antibacterial effect of monoglycerides and sucrose esters against *E. coli*. These combinations also were effective against other Gram-negative bacteria, such as *Serratia marcescens*, *Proteus vulgaris*, and *Salmonella* Typhimurium. These same workers reported the combined use of monolaurin, organic acids, and EDTA (Tsuchido et al., 1981). The enhanced bactericidal activity of monolaurin by acid or EDTA was the result of the increased uptake of the monoglyceride into the cells. Significant inhibition of amino acids occurred as a result of monolaurin treatment in the presence of citric acid. Individually, neither monolaurin nor citrate had any effect of their own on *E. coli*. Fatty acids and their esters were shown to have an effect on the thermal destruction of *E. coli* and *P. aeruginosa* (Kato and Shibasaki, 1975c). The enhancing effect by monolaurin was at a very low concentration of 100 ppm. Thus, lower heat treatments (improved food quality) may be possible using monolaurin in combination with other hurdles.

Stern et al. (1979) evaluated combinations of BHA, salt, and pH. They reported that BHA became more effective as the pH was reduced from 7.0 to 5.0 and the salt concentration increased from 3% to 7%. The combination of 100 ppm BHA with 5% or 7% sodium chloride at all pH values tested had the strongest antibacterial effect. A unique approach for studying various combinations of factors on monolaurin activity was described by Bal'a and Marshall (1996a). Their approach used the double-gradient plate technique to analyze the combined interaction of pH, salt, monolaurin, and temperature on growth of *L. monocytogenes*. Results of their work are shown in Figures 11.2 through 11.5, where monolaurin activity is clearly seen to increase with high salt and low pH. A clear graphic display of growth domains of the bacterium was produced showing the combined effect of the four variables (Figure 11.6).

All these GRAS agents are effective individually to various degrees, but all have limitations when used alone. Fatty acids and their derivatives are effective against Gram-positive bacteria and fungi but have little activity against Gram-negative bacteria. Phenolic antioxidants are active against

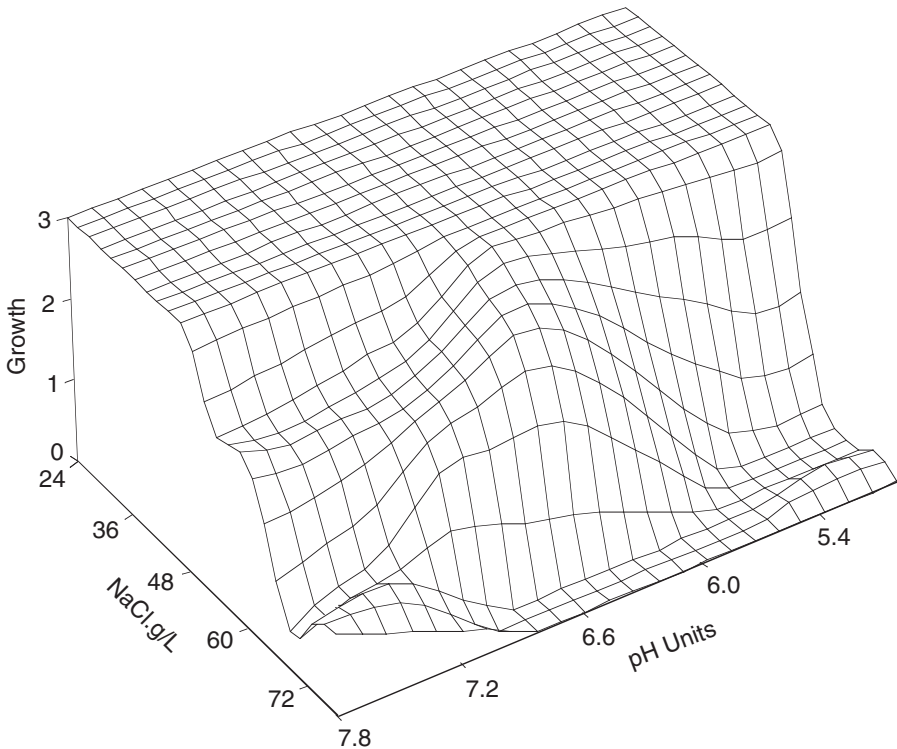


FIGURE 11.2 Growth profile of *Listeria monocytogenes* on two-dimensional salt and pH gradient plates incubated at 35°C for 48 hours in the presence of 0 µg of monolaurin per ml (From Bal'a and Marshall, 1996a).

a wide variety of microorganisms but are limited by toxicologic considerations. EDTA has little antimicrobial activity by itself and needs to be considered as a potentiator for preservative action rather than a biocide on its own merit (Kenward et al., 1979). To maximize the effectiveness of these in foods, it is necessary to adopt a systems approach (hurdle technology) to the challenge posed by the need for food preservation and to avoid the limitations of single chemical preservatives. The systems approach advantageously uses the multiple function of the food additives discussed and introduces them into food products to create an environment hostile to microorganisms. When BHA and EDTA are used, oxidative deterioration and microbial growth can be reduced or even eliminated. Such complete preservative systems require that food technologists have a good understanding of multifunctional additives and are able to describe the “where, when, and how much” for the optimal usage of these preservative additives. Thus, the food technologist should think in terms of a preservative system rather than a chemical *per se* to protect food from spoilage. The advantages of this approach are several:

1. Chemicals already approved for food are used.
2. The chemicals mentioned are well known to the industry, and therefore new technology for their introduction need not be developed.
3. The system becomes self preserving.

The preservative system approach not only solves a microbiological problem but also may help create a new marketing tool by deemphasizing the need for classic preservatives — that is, preservation without (classic) preservatives. For less developed countries the preservative systems philosophy removes or lowers the requirement for refrigerated storage of the food. A start has been made, and much more needs to be done to exploit these initial successes.

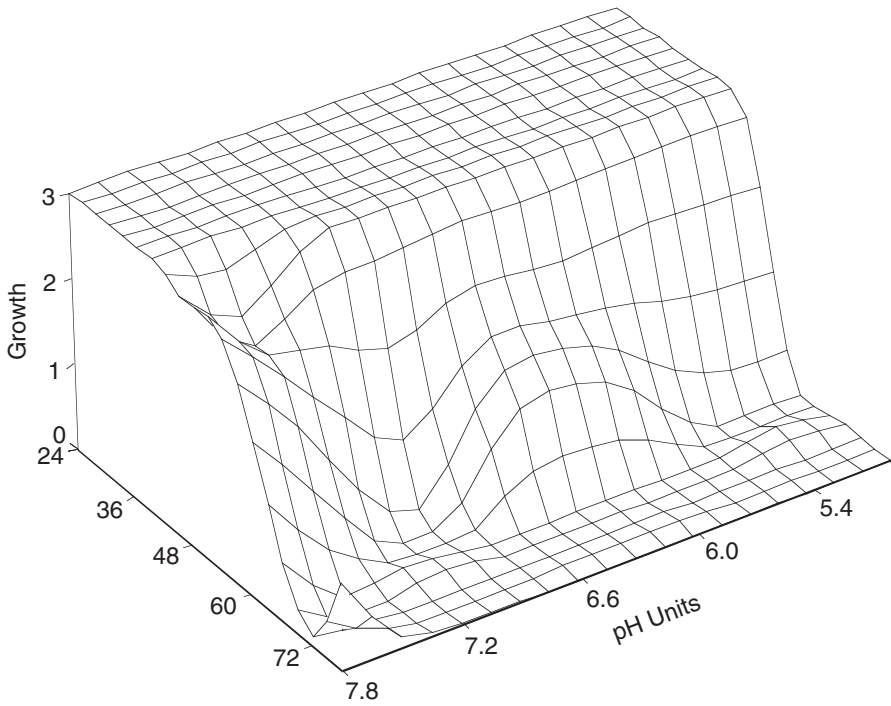


FIGURE 11.3 Growth profile of *Listeria monocytogenes* on two-dimensional salt and pH gradient plates incubated at 35°C for 48 hours in the presence of 2 µg of monolaurin per ml (From Bal'a and Marshall, 1996a).

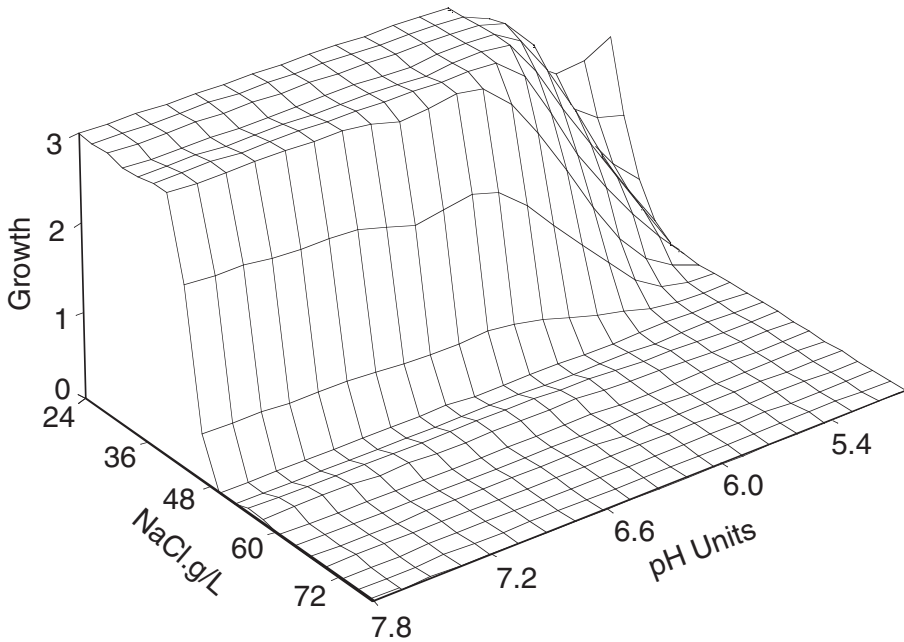


FIGURE 11.4 Growth profile of *Listeria monocytogenes* on two-dimensional salt and pH gradient plates incubated at 35°C for 48 hours in the presence of 4 µg of monolaurin per ml (From Bal'a and Marshall, 1996a).

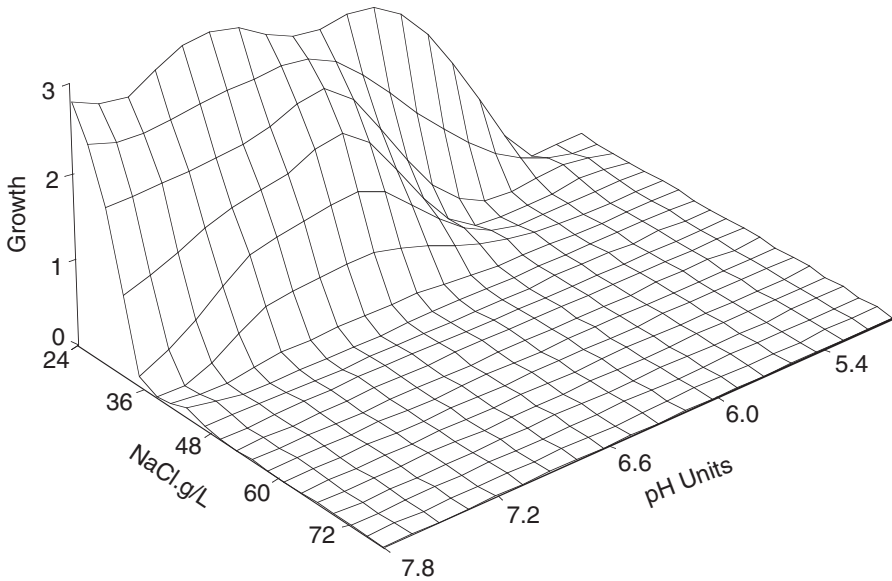


FIGURE 11.5 Growth profile of *Listeria monocytogenes* on two-dimensional salt and pH gradient plates incubated at 35°C for 48 hours in the presence of 8 µg of monolaurin per ml (From Bal'a and Marshall, 1996a).

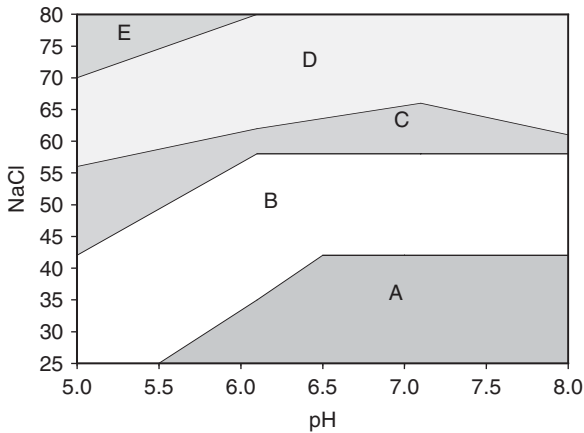


FIGURE 11.6 Effect of salt (g/L) and pH on growth domains of *Listeria monocytogenes* with monolaurin concentrations of (A) 8 µg/ml, (A+B) 4 µg/ml, (A+B+C) 2 µg/ml, (A+B+C+D) 0 µg/ml, and (E) area of no growth at 0 µg/ml (From Bal'a and Marshall, 1996a).

This chapter presents examples in which monolaurin, a GRAS emulsifier, has been used to create a hostile environment. Because the monoglyceride has multiple functions, it should not be considered a chemical preservative in the usual sense of the word.

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12 Lysozyme

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CONTENTS

Background and General Properties of Lysozyme.....	361
Properties of Lysozyme	362
Uses and Stability in Foods.....	366
Antimicrobial Spectrum of Activity	368
Effect of Lysozyme on Heat Resistance of Bacterial Spores	369
Activity of Lysozyme against Food-Related Organisms.....	370
Enhancement of Lysozyme Activity by Chemical Agents.....	371
Food Applications of Lysozyme.....	375
Chemically Modified Lysozyme Molecules as Antimicrobials	376
Nonenzymatic Antimicrobial Activity of Lysozyme and Lysozyme Peptides	377
Recombinant Lysozymes for Use as Antimicrobials in Foods.....	378
Regulatory Status and Toxicology.....	378
Summary and Perspectives	379
References	380

BACKGROUND AND GENERAL PROPERTIES OF LYSOZYME

Lysozyme was discovered in 1921 by Alexander Fleming (1881–1955), who described it as a “remarkable bacteriolytic principle” (Fleming, 1922). His subsequent discovery of penicillin occurred in the late 1920s. In recognition of his pioneering activities in antimicrobials and treatment of infectious diseases, Fleming was knighted in 1944 and shared the Nobel Prize in Physiology or Medicine in 1945 (MacFarlane, 1985). Fleming’s lysozyme research emerged from his demonstration that chemical antiseptics were ineffective in treating infections. He showed that his own nasal mucus inhibited the growth of a *Micrococcus* species, a fortuitous discovery because micrococci are among the most sensitive organisms to lysozyme. He first thought that the inhibitory factor was a bacteriophage, but later showed that it was an enzyme that lysed bacterial cells (Fleming, 1922). He established that lysozyme was an endogenous (innate) antimicrobial in the body, and his work supported the concept that an effective way to treat infections was to enhance the body’s own immune responses. Fleming found that lysozyme was present in nasal mucus and tears and that hen egg white had a particularly high level of the protein. It is noteworthy that Laschenko in 1909 and Rettger and Sperry in 1912 showed that egg white was capable of causing lysis of certain strains of bacteria and spores (Laschenko, 1909; Rettger and Sperry, 1912). It was Fleming, however, who actually isolated the lytic principle and showed that it occurred in human secretions.

Lysozyme was the first enzyme whose primary amino acid sequence was determined and was also the first enzyme whose structure was determined by X-ray crystallography (Blake et al., 1965; Phillips, 1966). It has an ellipsoid structure with dimensions of approximately of $45 \times 30 \times 30$ angstroms. Its catalytic activity was demonstrated in 1966 by John Rupley, who showed that lysozyme cleaved purified oligosaccharides of N-acetylglucosamine containing a β -(1-4) glycosidic

bond (Rupley, 1967). Oligosaccharides with more than three sugar units were hydrolyzed, and the hexasaccharide was shown to be the optimum length substrate. Several families of lysozyme have been found, but they all share the characteristic property of cleaving a β -glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 on N-acetylglucosamine of bacterial peptidoglycan.

Although traditionally associated with eggs of birds, especially those of domestic hens, lysozyme is widespread in nature and is found in many sources including certain vegetables, insects, plants, and fungi (Masschalck et al., 2001; Tranter, 1994; Jolles and Jolles, 1984). Lysozyme is present in human colostrum (Mathur et al., 1990) and mammalian tissues and fluids such as milk, saliva, mucus, blood, and tears. It is also present in high concentrations in macrophages, leukocytes, monocytes, and neutrophilic granulocytes. Lysozyme has important roles in the immune response of organisms in reaction to infections and inflammation (Osserman et al., 1974; Sava, 1996). The major families of naturally occurring lysozyme that differ markedly in amino acid primary structure and structure are as follows: C for chicken or classical, G for goose, bacterial lysozyme (autolysins), phage lysozyme, and plant lysozyme (Jolles, 1996), but only the C enzyme from the hen egg whites is currently used in food preservation. Approximately 3.5% of the total protein content in hen egg white is lysozyme (Alderton et al., 1945; Sofos et al., 1998; Yamamoto et al., 1997). Lysozyme is present at concentrations of about 0.1, 0.13, and 0.25 $\mu\text{g/ml}$ in milk from sheep, cows, and goats, respectively (Chandan et al., 1968), while human milk contains 0.4 $\mu\text{g/ml}$ lysozyme (Reiter, 1978). Lysozyme is considered to be one of the most important factors of nonspecific immunity in human breast milk (Hennart et al., 1991). It also occurs at much higher concentrations in colostrum than in milk and may have positive attributes on the intestinal flora of nursing infants (Barbara and Pellegrini, 1976). Lysozyme has been proposed for use in various clinical applications, including antibacterial, antiviral, and antiinflammatory treatments in humans and animal species (Sava, 1996; Proctor and Cunningham, 1988). As described in detail in the following sections, lysozyme is also used to control microbial growth in foods such as cheese and wine and has potential uses as a preservative in other food systems. Lysozyme serves as a good model of an ideal food preservative in many respects because it is an innate component of the human immune system and thus would be expected to have low toxicity; it is an enzyme that acts catalytically and can be used at low concentrations in foods; it is specific for bacterial peptidoglycan and does not react with human tissue; and it has certain desirable resistance properties to heat, pH, and other intrinsic and extrinsic factors of foods.

PROPERTIES OF LYSOZYME

Early researchers described various methods for the purification and assay of lysozyme from egg white (Alderton et al., 1945; Meyer et al., 1936). Purified commercial lysozyme for food use is currently produced from hen egg whites in high yield by cation exchange resins. Its high affinity to cation resins is the result of the basic character of the enzyme, its isoelectric point of 10.5 to 11.0, and its monomeric structure and molecular weight of $\sim 14,400$ Da (Cunningham et al., 1991). Lysozyme is eluted from the resins by NaCl and is recovered as the hydrochloride salt. In addition to removal of lysozyme from egg albumin, the process also removes more than 90% of the avidin from eggs. The extracted egg whites are not nutritionally or functionally changed and are approved for food uses. Lysozyme is generally used as the hydrochloride salt, which is freely soluble in water and buffers. Lysozyme hydrochloride appears as a white, fine powder and has a slightly sweet taste. A 2% lysozyme hydrochloride solution has a pH of about 3.3. It is insoluble in most organic salts and concentrated salt solutions but regains activity when transferred back to an aqueous solution. Depending on the pH conditions, lysozyme can polymerize (Sophianopoulos and Van Holde, 1964). The basic properties of lysozyme are described in Table 12.1.

Lysozyme (E.C. 3.2.1.17) is classified as a mucopeptide N-acetylmuramyl hydrolase by the Commission on Enzymes (Connor, 1993) and is commonly referred to as muramidase (Proctor and Cunningham, 1988). The primary amino acid sequence and structure as determined by X-ray

TABLE 12.1
Properties and Specifications of Food-Grade Lysozyme Hydrochloride for Use in the United States^a

a. Generally recognized as safe substance:	Egg-white lysozyme (CAS Reg. No. 9001-62-2) obtained by extraction of egg whites.
b. Enzyme property:	Peptidoglycan N-acetylmuramoyl hydrolase (EC No. 3.2.1.17) that catalyzes the hydrolysis of peptidoglycan in the cell walls of certain bacteria. The ingredient is used as an enzyme as defined in Sec. 170(o)(9) in 21 CFR Part 184.
c. Primary food application:	Cheeses as defined in 1 CFR, Part 51, Sec. 170.3(n)(5), in accordance with Sec. 184.1(b)(3). The primary enzyme targets are germinating spores of <i>Clostridium tyrobutyricum</i> , an organism responsible for late blowing of certain cheese varieties.
d. Use level:	Levels not to exceed current good manufacturing practices in cheese.
e. Label requirement:	Bulk and packaged foods that contains cheese manufactured using egg-white lysozyme shall include the usual name "egg-white lysozyme" on its ingredient label.
f. Commercial ingredient:	Food grade lysozyme hydrochloride.
g. Food use properties:	Freely soluble in water; pH 3.3; electrophoretically pure.
h. Limit specifications:	Satisfies limit tests and regulations for heavy metals, arsenic, and microbial limit tests. According to IDF (1987) salmonellae absent in 25 g; <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> , <i>E. coli</i> ; sulfite-reducing clostridia absent in 1 g; Coliforms max 30/g.

^a Sources include: International Dairy Federation 1987; U.S. FDA 1998; <http://www.fordras.com>; <http://www.inovatech.com>. For regulatory information for other countries, the manufacturer is advised to contact the major manufacturers of lysozyme, whose Web sites are listed here.

crystallography of hen egg-white lysozyme have been described in detail (Blake et al., 1965; Phillips, 1966; Jolles et al., 1963; Rees and Offord, 1972). Egg-white lysozyme is a single polypeptide chain of 129 amino acids cross-linked by four disulfide bridges (Jolles et al., 1963; Jolles, 1996; Phillips, 1966) with a molecular weight of approximately 14,400 Da (Barbara and Pellegrini, 1976; Jolles and Jolles, 1984). Enzymatic activity of the molecule is lost if at least two of the disulfide bridges are not intact, or if all disulfide bonds are reduced (Proctor and Cunningham, 1988; Wang and Shelef, 1992). Lysozyme has a hydrophobic core with hydrophilic amino acid side chains toward the surface, giving the molecule stability (Proctor and Cunningham, 1988).

The lytic activity of lysozyme has traditionally been measured by observing lysis of ultraviolet-killed and lyophilized *Micrococcus luteus* (*M. lysodeikticus*) cells based on the early observations of Fleming, and this assay has been optimized by Shugar (Fleming, 1922; Parry et al., 1969; Shugar, 1952). Numerous variations for assay of lysozyme by turbidimetry have been reported (detailed in Proctor and Cunningham, 1988). One unit of lysozyme is defined as the smallest quantity leading to complete lysis of *M. luteus* in a serial dilution test (Alderton et al., 1945). Specifically, one Shugar unit is that quantity of enzyme in 1 ml of a suspension of *M. luteus* inactivated cells at pH 7.0, with an initial absorbance of 0.750 at 450 nm in a pathlength of 1 cm, which causes the absorbance to decrease at a rate of 0.001 per minute. The maximal specific activity of lysozyme at 25°C using *M. luteus* as the substrate generally is ~50,000 units/mg, but it can be much less (~20,000 units/mg) depending on the lysozyme preparation. From a practical perspective, it is often desirable to use a wavelength of 650 nm to reduce absorption by colored substances in food extracts. Certain compounds can inhibit the lysozyme assay including surfactants such as sodium dodecyl sulfate, iodine, and fatty acids, and alcohols of C12 or higher (Smith and Stroker, 1949). Several

alternate methods of assay have been developed including chromatographic separations, agar plate lysis, radioimmunoassays, and fluorescence systems, but these methods are generally only used for special purposes. The *M. luteus* lysis method is the standard (Proctor and Cunningham, 1988; Jolles and Jolles, 1984). A sensitive reversed-phase high-performance liquid chromatography (HPLC) detection method for lysozyme was developed in 2000 for use in milk and cheese samples (Pellegrino and Tirelli, 2000).

The optimum pH for lysis of *Micrococcus lysodeikticus* *in vitro* by egg-white lysozyme is ~6.6 (Barbara and Pellegrini, 1976). Enzymatic activity is observed over the pH range of 3.5 to 7.0 (Wang and Shelef, 1992), although lower activity has been observed at pHs below 3.5 or above 7.0 (Smolelis and Hartsell, 1952; Yang and Cunningham, 1993). The optimum pH for activity is strongly dependent on salt concentration (Chang and Carr, 1971; Davies et al., 1969). At pH values below 5.3, reaction products can inhibit the enzyme (Barbara and Pellegrini, 1976). Lysozyme is active *in vitro* from 1°C to near-boiling temperature (Johnson, 1994).

The principal natural substrate of lysozyme is the peptidoglycan of bacteria cell walls, in which the polysaccharide is comprised of alternating β -(1-4) linked N-acetyl glucosamine and N-acetylmuramic acid residues (Salton, 1957; Sharon, 1967). Lysozyme targets the peptidoglycan layer of bacterial cell walls, hydrolyzing the β -(1-4) linkage between N-acetylmuramic acid and N-acetylglucosamine, resulting in cell lysis (Proctor and Cunningham, 1988; Pakkanen and Aalto, 1997; Salton, 1957). Lysozyme also has weaker enzymatic activity on chitin, such as occurs in the bud scars of certain yeasts and in the cell walls of filamentous fungi. Lysozyme is most active on certain Gram-positive bacteria, particularly *M. luteus* (*M. lysodeikticus*), which is used for assay of activity, and is also highly active on certain clostridia, bacilli, and other Gram-positive bacteria (Table 12.2). Lysozyme activity varies widely among Gram-positive bacteria. For example, in its application for prevention of late blowing by spores of *Clostridium tyrobutyricum* (International Dairy Federation, 1987), the lactic acid starter cultures are generally not affected (Bester and Lombard, 1990; Wasserfall, 1978). Lysozyme is inactive against certain Gram-positive bacteria, such as *Staphylococcus aureus* in which the amino group on the peptidoglycan is modified by chemical groups, or on certain species containing large capsules. Lysozyme has weak or negligible activity on Gram-negative bacteria, probably mainly because of its inability to penetrate the outer membrane. However, being strongly cationic, lysozyme also reacts with negatively charged components including nucleic acids, certain proteins, and lipopolysaccharide with some loss of activity (Cunningham and Lineweaver, 1967; Giangiacoimo et al., 1992; Ohno and Morrison, 1989). Under most conditions encountered in foods, the lipopolysaccharide layer in the outer membrane of Gram-negative bacteria offers protection from lysozyme through a combination of negative charge and cross bridging with divalent cations (Hancock, 1984; Nikaido, 2001; Nikaido and Vaara, 1985). However, disruption of the outer membrane by physical or chemical treatments such as the addition of ethylenediamine tetraacetic acid (EDTA) can increase the sensitivity of Gram-negative bacteria to lysozyme. A “rough” mutant of *Salmonella enterica* serotype Typhimurium with a defect in the lipopolysaccharide (LPS) component of the cell wall was inhibited by lysozyme without EDTA treatment, unlike “smooth” strains, which required the presence of EDTA for inhibition (Sanderson et al., 1974). As a result of its basic character, lysozyme can cause flocculation of various bacteria or toxins (Barbara and Pellegrini, 1976).

Lysozymes from different organisms have the same substrate specificity and catalytic activity (U.S. FDA, 1998). However, depending on the evolutionary lineage of lysozymes (e.g., types C, G, or phage), the biochemical properties are different. The amino acid content of bovine milk lysozyme is different than that of human milk lysozyme or egg-white lysozyme (Eitenmiller et al., 1976). The optimum pH for activity of bovine milk lysozyme is 7.9 (Chandan et al., 1965). Bovine milk lysozyme contains 154 amino acids and has a molecular weight around 18,000 (Eitenmiller et al., 1976). Bovine milk lysozyme is more heat stable than egg-white lysozyme at pH 4 but less

TABLE 12.2
Antimicrobial Spectrum of Egg-White Lysozyme against Food-Related Pathogens and Spoilage Organisms

Organisms Strongly Lysed or Inhibited

Bacillus coagulans
Bacillus stearothermophilus
Clostridium thermosaccharolyticum
Clostridium tyrobutyricum
Micrococcus spp.
Sarcina spp.

Organisms Moderately Inhibited or Showing Marked Strain Sensitivity

Bacillus cereus
Brucella spp.
Campylobacter jejuni
Clostridium botulinum serotypes types A, B, and E
Listeria monocytogenes
Enterococcus faecalis
Lactobacillus spp.
Moraxella spp.
Pseudomonas aeruginosa
Yersinia enterocolitica
 Yeasts—*Candida albicans*, *Cryptococcus neoformans*, select others

Organisms Usually Not Lysed or Inhibited

Aeromonas hydrophila
Brocothrix thermosphacta
Clostridium butyricum
Clostridium perfringens
Escherichia coli O157:H7
Klebsiella pneumoniae
Lactococcus spp.
Leuconostoc spp.
Salmonella enterica serotype Typhimurium
Shewanella putrefaciens
Shigella spp.
Staphylococcus aureus
Streptococcus thermophilus and other starter streptococci
Vibrio cholerae

Note: The data included in this table are collected from a variety of sources including primary sources: Barbara and Pellegrini 1976; Cunningham et al., 1991; Hughey and Johnson 1987; Salton 1957; Wooley and Blue 1974; and other papers cited in the text of this chapter. It should be noted that many of the resistance organisms can be inhibited or lysed when certain physical or chemical treatments are used in conjunction with lysozyme. Also, these data are mostly from *in vitro* experiments and do not represent activity of lysozyme against food-related organisms in food systems. These aspects are discussed in the text.

heat stable at pH 7 or 9 (Eitenmiller et al., 1976). Lysozymes found in plants often exhibit weak chitinase activity to protect plant tissues by hydrolyzing the β -(1-4) linkages binding N-acetylglucosamine molecules of chitin found in certain fungi (Jolles et al., 1963; Jolles and Jolles, 1984). Some lysozymes also exhibit esterase activity (Tranter, 1994; Jolles and Jolles, 1984).

USES AND STABILITY IN FOODS

Lysozymes have several desirable biochemical properties for use as food preservatives. They are generally stable under a variety of conditions, including those typically found in many minimally processed foods. Lysozyme in Camembert cheese was detected for several weeks (Hughey et al., 1989). Lysozyme is stable in certain cheeses for months to years because it effectively prevents late blowing, which commences during long-term aging of the cheese (International Dairy Federation, 1987). Lysozyme (10 to 20 $\mu\text{g/ml}$) was stable in sweetened sake (mirin) containing less than 20% alcohol for 20 weeks at 37°C (Yashitake and Shinichiro, 1977), for 1 year at room temperature (Uchida et al., 1972), and during pasteurization at 65°C for 1 to 5 hours (Uchida et al., 1972; Yashitake and Shinichiro, 1977). Lysozyme activity can be inhibited by certain surfactants, fatty acids of C12, certain ions, and LPS. During processing, it can also react with proteins, probably mainly forming mixed disulfides with itself or other proteins or by ionic interactions. Lysozyme is stable in many acidic solutions (Wang and Shelef, 1991) but has limited stability under alkaline conditions (Alderton et al., 1945). Lysozyme can be frozen (Sofos et al., 1998) and is stable in dry storage at 5°C for several years (Wilkinson and Dorrington, 1975). Lysozyme was more stable in stored, spray-dried, whole-egg powder stored at 0°C than 37°C, although lytic activity was still present after 3 months even at the higher temperature (Hartsell, 1948). When considered for a food application, the stability of lysozyme should be tested in the food system.

Lysozyme is among the most heat-stable enzymes and can resist boiling for 1 to 2 minutes at pH 4 to 5, but it is denatured by heat at higher pHs (Ahern and Klibanov, 1985; Sofos et al., 1998). Irreversible denaturation at 100°C occurred much slower at pH 4 than at pH 8 (Ahern and Klibanov, 1985). Heating for 5 minutes at 100°C at pH 9.0 resulted in loss of most activity (Meyer et al., 1936). Lysozyme is usually not affected by temperatures up to 55°C, although stability during heating varies with the food system, pH, and salt concentrations (Wang and Shelef, 1992). Heat stability of lysozyme decreased above pH 7.0 (Smolelis and Hartsell, 1952, Wang and Shelef, 1991), with much greater stability at pH 7.0 than pH 9.0 in egg white (Cunningham and Lineweaver, 1965), probably because of the reduction of at least one disulfide bond by the sulfhydryl group of ovalbumin (Cunningham and Lineweaver, 1967). At pH 6.2, egg-white lysozyme lost only 5% of lytic activity after heating at 80°C for 30 minutes and lost 25% of activity after heating at 100°C for 20 minutes (Smolelis and Hartsell, 1952). Heat stability between 85°C and 95°C was highest at approximately pH 5.5 (Beychok and Warner, 1959).

Inactivation of lysozyme occurs during heating in certain foods. In skim milk, activity decreased at temperatures above 60°C (Weaver and Kroger, 1978). Nonetheless, sufficient activity remains after pasteurization of milk to prevent late blowing of cheeses by *C. tyrobutyricum*. At high temperatures, it is likely that lysozyme can bind to and become inactivated by proteins in certain foods. The heat stability of lysozyme is dependent on food type (Gould, 2002). No loss of lysozyme activity occurred in egg white heated at 60°C for 60 minutes at pH 5 (Sandow, 1926). Enzymatic activity of lysozyme remained stable in camel, cow, and buffalo milk samples heated to 65°C for 30 minutes, although activity was reduced 56% to 81% and 94% to 100% in milk samples heated for 30 minutes at 85°C or 100°C, respectively (Elagamy, 2000). Extremely high temperatures such as those used in roasting can inactivate lysozyme. After dry heating at 150°C for 20 minutes, polymerization through disulfide linkages was observed, and both polymerization and degradation were noted after heating at 180°C for 20 minutes (Hayase et al., 1975). However, some enzymatic activity remained after treatment at both temperatures (Hayase et al., 1975). At a higher temperature (250°C), decomposition of almost all amino acids in the polypeptide occurred (Fujimaki et al., 1972). The development of stable formulations of lysozyme for use in foods that receive a heat treatment would increase the utility of the enzyme. Sugars and polyols can protect lysozyme against heat denaturation, possibly as a result of the stabilizing effect of the compounds on hydrophobic interactions (Back et al., 1979). For instance, sucrose

increased thermal stability of lysozyme, especially with a decrease in pH from 6.0 to 3.2 (Yashitake and Shinichiro, 1977).

Nonthermal treatments used in food processing can have a deleterious effect on lysozyme. Pulsed electric field treatment of lysozyme in buffer resulted in a 15% to 60% loss of enzymatic activity compared to untreated controls (Ho et al., 1997). Inactivation of lysozyme by ultraviolet radiation follows first-order kinetics (Shugar, 1952). Egg-white lysozyme, bovine milk lysozyme, and human milk lysozyme were sensitive to >10 Krad gamma irradiation at ambient temperature in phosphate buffer at pH 6.2, although inactivation was concentration dependent (Eitenmiller et al., 1971). Inactivation of lysozymes by gamma irradiation was the result in part of hydroxyl radicals (Eitenmiller et al., 1971). Kume and others (1973) observed a 37% inactivation of lysozyme activity in aqueous solution by 0.35 Mrad gamma irradiation, although only slight inactivation of lysozyme occurred in irradiated egg white treated under the same conditions, possibly as a result of protection by high protein levels in the egg white. Results of another study showed that both purified lysozyme in buffer and endogenous lysozyme in egg white were not significantly affected by 19.5 Krad gamma irradiation at 60°C (Schaffner et al., 1989). Gamma irradiation at 2 Mrad polymerized lysozyme through covalent bonds, especially in the presence of peroxidizing lipids and at higher water activity levels (Kanner and Karel, 1976).

Lysozyme retains activity in the presence of many common components and compounds inherently present or added to foods. Activity of lysozyme was not affected by the presence of certain antimicrobial compounds used in foods, including sodium nitrite, ethanol, butylated hydroxyanisole, calcium propionate, potassium sorbate, and propyl paraben (Yang and Cunningham, 1993). Potassium dichromate had no effect on lysozyme activity in mastitic bovine milk (Weaver and Kroger, 1978). Some organic solvents used in foods, such as ethanol, do not denature lysozyme (Sofos et al., 1998).

Certain chemicals that may be present in foods can have a detrimental effect on lysozyme activity. Lysozyme can bind to certain food components, leading to a loss in activity (Cunningham and Lineweaver, 1967; Sofos et al., 1998). Lysozyme may be less heat stable in proteinaceous foods than *in vitro* as a result of the formation of mixed disulfide-linked molecules or other interactions with proteins or other constituents in the food (Johnson, 1994). Purified lysozyme in buffer at pH 6.2 was inactivated by egg yolk (Cunningham and Cotterill, 1971), possibly as a result of electrostatic interaction between lysozyme and substances in the yolk (Proctor and Cunningham, 1988). Activity was lost in the presence of high concentrations of lactic acid, acetic acid, and chlorine (Yang and Cunningham, 1993). Lysozyme can also be inhibited by substances such as alkyl sulfates, aliphatic long-chain alcohols, and fatty acids and their sodium salts (Smith and Stroker, 1949). Polysaccharides with carboxylic acid and sulfonic acid groups, such as pectin and alginate, also reduced lysozyme activity, although addition of 1% to 6% salt minimized inactivation by these compounds (Yashitake and Shinichiro, 1977). High concentrations of polyvalent and divalent cations such as cobalt, manganese, mercury, and copper — as well as low concentrations of iodine — can inactivate lysozyme (Proctor and Cunningham, 1988).

Salt level plays a critical role in the enzymatic activity of lysozyme. Nonspecific activation occurs at low salt concentrations (Tranter, 1994; Chang and Carr, 1971), although lysozyme activity is inhibited at high salt concentrations greater than 0.05 to 0.1 M (Chang and Carr, 1971; Davies et al., 1969; Smolelis and Hartsell, 1952). The addition of 3 mM NaCl improved the lysis of rapidly growing sensitive organisms by lysozyme (Vakil et al., 1969). Inhibition of lysozyme activity in high ionic strength solutions was stronger at alkaline pH values (Davies et al., 1969). The presence of sodium nitrate (180 mg/L) or sodium chloride (270 mg/L) did not inhibit the activity of lysozyme (450 mg/L) against several Gram-positive and Gram-negative bacteria under nongrowth-restricted conditions (Gill and Holley, 2002).

ANTIMICROBIAL SPECTRUM OF ACTIVITY

Lysozyme exhibits antimicrobial activity against vegetative cells of a wide variety of organisms, including numerous foodborne pathogens and spoilage organisms. Egg-white lysozyme is generally most active against certain Gram-positive organisms and is ineffective against dormant bacterial spores and Gram-negative bacteria. Antimicrobial activity of the enzyme can vary widely within groups of similar organisms, and its lytic activity is dependent on growth medium, test conditions, age of cells, presence of interfering substances, and many other parameters.

Gram-positive bacteria vary markedly in their sensitivity to lysozyme depending on the strain, species, and conditions. Spoilage of foods by Gram-positive organisms including heterofermentative lactics, leuconostocs, staphylococci, *Brochothrix*, and certain other Gram-positive bacteria is a major problem in the food industry, but these groups of organisms are mostly resistant to the enzyme. Species of the Gram-positive bacteria *Micrococcus* and *Sarcina* species are generally more sensitive to lysozyme than are *Lactobacillus* and *Bacillus* species (Connor, 1993). Ashton and others (1975) demonstrated that prevention of growth of *B. stearothermophilus* and *B. coagulans* by egg albumin was the result of the presence of lysozyme. Human lysozyme inhibited *Staphylococcus aureus* (Maga et al., 1998), but the egg-white enzyme is generally inactive by itself against *S. aureus*. Lysozyme susceptibility among species of Gram-positive bacteria may decrease by derivatization of the amino groups and may vary because of differences in the relative proportions of N-acetyl-amino sugars in the cell walls (Salton and Pavlik, 1960). Specifically, higher proportions of β -1-6 and β -1-3 linkages between N-acetylmuramic acid and N-acetylglucosamine are found in the peptidoglycan of some Gram-positive bacterial species, which are more resistant to lysozyme than β -1-4 linkages. These differences in cell-surface structure contribute to lysozyme susceptibility in the Gram-positives (Vakil et al., 1969). Bacterial spores can be sensitized to lysozyme by strong reducing agents or oxidizing agents to rupture disulfide bonds (Gould and Hitchins, 1963), but these methods are not compatible with food use (Gould, 2002).

Gram-negative bacteria are generally less sensitive than Gram-positive bacteria to lysozyme mainly as a result of protection of the cell wall by the outer membrane. Resistant organisms include the important pathogens *Salmonella* species and *Escherichia coli* O157:H7. However, certain physiologic conditions, physical treatments, or chemicals that disrupt or alter the outer membrane can enhance activity of lysozyme against Gram-negatives. At pH 3.5, most Gram-negative bacteria tested in one study exhibited some sensitivity to lysis by lysozyme, especially *Salmonella* and *Brucella* species (Peterson and Hartsell, 1955). In another study, human and bovine milk lysozymes displayed activity against five Gram-negative bacteria, including some strains of Gram-negative bacteria that are resistant to egg-white lysozyme (Vakil et al., 1969), demonstrating variability in antimicrobial activity between lysozymes isolated from different sources. The susceptibility of various species of food-related organisms is summarized in Table 12.2 and represents the authors' assessment of the available literature. However, as discussed throughout this chapter, resistant organisms can often be made sensitive by physical treatments or chemical agents that disrupt outer membrane layers and capsules, allowing penetration of lysozyme and consequent lytic activity. Thus, in evaluating the potential for lysozyme function as a preservative in foods, a systems-based approach is often desirable whereby combinations of physical treatment or chemical agents are used in combination with lysozyme.

In a survey of the sensitivity of food-related bacterial pathogens and spoilage organisms to lysozyme (Hughey and Johnson, 1987), several organisms were susceptible to lysis and/or growth inhibition by 10 to 200 mg/L egg-white lysozyme in media and buffer systems, including *Listeria monocytogenes*, some strains of *Clostridium botulinum*, *C. thermosaccharolyticum*, *B. stearothermophilus*, and *C. tyrobutyricum*. In the same study, *B. cereus*, *C. perfringens*, *S. aureus*, *Campylobacter jejuni*, *E. coli* O157:H7, *Salmonella enterica* serotype Typhimurium, *Yersinia enterocolitica*, and other strains of *C. botulinum* were resistant to lysozyme.

Sensitivity of organisms to lysozyme may be dependent on physiologic state. Most studies examining the effectiveness of lysozyme have tested cells in nutrient-deficient environments. Cell-wall synthesis in rapidly growing cultures may exceed the rate of degradation by lysozyme (Hughey and Johnson, 1987). Lysozyme–EDTA combinations were less effective against rapidly growing Gram-negative organisms in broth media than in buffer (Gill and Holley, 2002). The antimicrobial effect of lysozyme and nisin against lactic acid bacteria was sometimes enhanced when the growth media was diluted (Chung and Hancock, 2000). Inhibition of growth of *L. monocytogenes* in culture media by lysozyme at 5°C and 25°C increased when the pH was lowered from 7.2 to 5.5, although lysis of nongrowing cells in buffer by lysozyme was not increased by a reduction in pH (Johansen et al., 1994). In that study, lower pH may have inhibited growth of the organism enough to allow the rate of hydrolysis by lysozyme to exceed the growth rate (Johansen et al., 1994). Heat-treated cells of *Yersinia enterocolitica* grown at 4°C were resistant to lysozyme, but heat-treated cells grown at 37°C were sensitive (Pagan et al., 1999). However, the activity of lysozyme against Gram-positive organisms was enhanced by EDTA under nongrowth-restricted conditions (Gill and Holley, 2002). During evaluation of the antimicrobial efficacy of lysozyme and lysozyme–antimicrobial combinations, testing of both growing and nongrowing cells may be advantageous.

EFFECT OF LYSOZYME ON HEAT RESISTANCE OF BACTERIAL SPORES

The safety of minimally processed, extended shelf life foods, including sous-vide and cook-chill products, is usually the result of a minimal heat treatment to achieve a 6-log reduction in heat-sensitive nonproteolytic *Clostridium botulinum* spores, followed by refrigeration below 10°C (Gould, 1999). However, heat treatment guidelines at 65°C to 95°C do not usually take into consideration the effect of lysozyme on spore heat resistance (Fernandez and Peck, 1999). Lysozyme, which is naturally present in many foods, is relatively heat resistant and may be active in foods after mild heat processes such as pasteurization (Peck et al., 1992).

At concentrations as low as 0.1 µg/ml (Peck et al., 1992) lysozyme increases the apparent heat resistance of nonproteolytic *C. botulinum* spores (Alderton et al., 1974; Fernandez and Peck, 1999). Increased heat resistance of proteolytic type A *C. botulinum* spores has been shown, although the effect was minimal compared to the effect on type E nonproteolytic spores (Alderton et al., 1974). Lysozyme also increased the recovery of heat-treated spores of *Clostridium perfringens* (Adams, 1974), and possibly *Bacillus cereus* (Blocher and Busta, 1982), but not *Bacillus thuringiensis* (Faille et al., 1999). Approximately 0.1% to 20% of nonproteolytic *C. botulinum* spores are permeable to lysozyme, resulting in a biphasic survival curve on heating (Peck et al., 1993). Lysozyme in the recovery medium is thought to compensate for a heat-damaged germination system (Broda et al., 1998) in lysozyme-permeable heat-sensitive bacterial spores (Peck et al., 1992), probably by degrading the spore cortex, allowing core hydration, and inducing spore germination (Gould, 1989).

Vegetable juice extracts with measurable lytic activity when added to the recovery medium increased the measurable heat resistance of nonproteolytic *C. botulinum* (Stringer and Peck, 1996). In other research, heating in a meat medium resulted in a 6-log reduction in numbers of viable nonproteolytic *C. botulinum* in the absence of lysozyme but not with lysozyme present (Peck and Fernandez, 1995). It is unknown whether the unusually high measured heat resistance of type E *C. botulinum* in Dungeness crab meat (Peterson et al., 1997) was because of the presence of lysozyme in the product.

Predictive models for the inactivation of nonproteolytic *C. botulinum* in minimally processed refrigerated foods in the presence of lysozyme have been developed (Fernandez and Peck, 1999). Because inadequate heat treatments may lead to subsequent growth of the organism during refrigerated storage, additional research is needed to verify the ability of currently used heat treatments

to adequately kill nonproteolytic *C. botulinum* in such foods containing lysozyme or similar lytic enzymes.

ACTIVITY OF LYSOZYME AGAINST FOOD-RELATED ORGANISMS

In this section we describe the effects of lysozyme on food-related organisms in selected studies. *Salmonella enterica* serotype Senftenberg was not detected on inoculated turkey drumsticks treated with 0.1% lysozyme for 3 hours at 22°C or 0.05% lysozyme for 1 minute at 63°C (Teotia and Miller, 1975). The heat resistances of *Salmonella enterica* serotype Enteritidis, *Salmonella enterica* serotype Typhimurium, *E. coli* O157:H7, and *Staphylococcus aureus* were significantly reduced in buffer in the presence of eggshell membranes containing lysozyme (Poland and Sheldon, 2001). In this system, components of the albumin may have enhanced the activity of enzyme against these organisms.

The antimicrobial activity of lysozyme has been extensively tested against *Listeria monocytogenes*, a Gram-positive pathogen of great concern to the food industry. Lysozyme (>2 mg/ml) prevented growth of *L. monocytogenes* in culture media at pH 7.0, and 2 mg/ml had listericidal effects at pH 9.0 (Wang and Shelef, 1991). Lysozyme was bactericidal to *L. monocytogenes* in milk, mozzarella, taleggio, and grana cheeses; in frankfurters; and on the surface of chickens (Dell'Acqua et al., 1989). Poor survival of the organism in egg white (Sofos et al., 1998; Erickson and Jenkins, 1992) is probably the result of the presence of lysozyme. Lysozyme was bactericidal to *L. monocytogenes* in egg-white-containing cholesterol-free mayonnaise (Erickson and Jenkins, 1991) and may be partially responsible for the rapid inactivation of *E. coli* O157:H7 in commercial mayonnaise made with whole eggs (Raghubeer et al., 1995). Pretreatment of raw cod-fish fillets with 3 mg/ml lysozyme delayed growth of *L. monocytogenes* at abuse temperature and was listericidal at refrigeration temperature, although lysozyme did not affect growth of the natural microflora in the product (Wang and Shelef, 1992). Egg-white lysozyme alone (100 ppm) killed or prevented growth of *L. monocytogenes* in several foods, particularly vegetables (Hughey et al., 1989). Lysozyme in Camembert cheese initially was bactericidal to *L. monocytogenes*, although the organism eventually grew in the cheese after 35 days (Hughey et al., 1989). *L. monocytogenes* cells grown at low temperature are more sensitive to lysozyme than cells grown at ambient temperature (Johnson, 1994) or 37°C (Smith et al., 1991), indicating that lysozyme may be useful for controlling the organism in refrigerated foods. Milk pasteurization in combination with added lysozyme may eliminate *L. monocytogenes* in certain cheeses prepared from milk contaminated with the organism. Numbers of heat-stressed *L. monocytogenes* significantly decreased in Cheddar-type and Camembert-type cheeses containing lysozyme (Johnson, 1994).

However, growth of *L. monocytogenes* was not inhibited on the surface of frankfurters formulated with 0.01% lysozyme (Bedie et al., 2001). Lysozyme had limited effectiveness against *L. monocytogenes* in pork sausage (Hughey et al., 1989) and pork, beef, or turkey frankfurters (Johnson, 1994). *L. monocytogenes* was resistant to hen egg-white lysozyme in milk (Hughey et al., 1989; Kihm et al., 1994) but sensitive to the enzyme in media or buffer, or in mineralized milk during heating (Kihm et al., 1994). Mineral or mineral-associated components in milk may protect the organisms from inactivation by lysozyme and heat in milk, probably by increasing the stability of the cell surface (Kihm et al., 1994).

The effect of lysozyme against clostridial species has been well documented. Spores of *Clostridium tyrobutyricum* survive milk pasteurization and cause "late blowing" of cheeses such as Edam and Gouda during ripening, which results in cracks or fissures in the finished cheeses. Lysozyme lyses *C. tyrobutyricum* cells as they outgrow from germinated spores and can prevent blowing of cheeses through lactate utilization by the organism (Carini and Lodi, 1982). The effectiveness of lysozyme used in the prevention of cheese blowing varies with the overall bacteriologic quality of the cheese milk used (Luck and Jager, 1997). Milk varies in spore content depending on the geographic location of the dairy, the season, and the feed. Spores are often much higher in cheese

milk obtained from cows fed silage in the winter months. Late blowing by spore formers in Edam cheese prepared with low-quality milk (i.e., high sore load) was inhibited by 500 units/ml lysozyme (Wasserfall et al., 1976). Growth of *C. tyrobutyricum* in Italian cheeses was also inhibited by 50 ppm lysozyme or nontoxic lysozyme salts added to butter or cheese (Ferrari and Dell'Acqua, 1979). Lysozyme in culture media inhibited growth of some bacteria associated with Gouda cheese, including *C. tyrobutyricum*, some *Lactobacillus* isolates, and coliforms (Bester and Lombard, 1990). Vegetative cells of *C. tyrobutyricum* can develop resistance to lysozyme (Wasserfall and Teuber, 1979). However, lysozyme prevented growth of the organism in Edam cheese, even when spores from lysozyme-resistant vegetative cells were used to contaminate the cheese milk (Wasserfall and Prokopek, 1978). In one study using *C. tyrobutyricum* spores that were resistant to lysozyme, subsequent proliferation of vegetative cells after spore germination was severely inhibited by lysozyme (Wasserfall and Teuber, 1979).

Lysozyme is also effective against several other clostridial species and may be useful to prevent growth of *C. botulinum* in foods. Egg-white lysozyme was antimicrobial to *C. botulinum* in foods such as turkey, pork sausage, salmon, asparagus, potatoes, tomatoes, and mushrooms (Johnson, 1994; Sofos et al., 1998). Lysozyme also delayed botulinum toxin production in bratwurst and salmon and in cod suspensions (Johnson, 1994). Lysis of *C. botulinum* by lysozyme in culture medium was enhanced at lower temperature and pH (Johnson, 1994).

Lysozyme inhibition of lactic acid bacteria, including many starter cultures used in foods as well as spoilage organisms, is variable among species. Lysozyme in milk is usually more inhibitory to pathogenic and spoilage organisms than to lactic acid bacteria (Cunningham et al., 1991). In culture media, lysozyme had no effect on the growth of Gouda cheese starter cultures (Bester and Lombard, 1990). Another study demonstrated inhibition of one streptococci starter culture from semihard cheese (Wasserfall, 1978). Spoilage lactobacilli were inhibited by 20 ppm lysozyme in sweetened sake (Uchida et al., 1972). *Lactobacillus delbrueckii* subspecies *lactis* was sensitive to ≥ 0.5 mg/L lysozyme in culture media, although susceptibility of the organism to the bacteriocin acidocin was reduced in the presence of lysozyme (Chumchalova et al., 1998). The application of lysozyme did not affect growth of lactic acid bacteria or yeasts on ready-to-use pear cubes, demonstrating that the action of lysozyme may be affected by substrate (Pittia et al., 1999).

Chitinase activity exhibited by lysozyme is probably responsible for the weak activity of lysozyme against several pathogenic and spoilage yeasts, including *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces rouxii* (Johnson, 1994). Lysozyme inhibits growth of spoilage lactic acid bacteria in wine without affecting growth of wine yeast, and this is a growing application of lysozyme in foods (Gerbaux et al., 1997).

ENHANCEMENT OF LYSOZYME ACTIVITY BY CHEMICAL AGENTS

The outer membrane of most Gram-negative bacteria protects the cell wall from lysozyme and may limit the effectiveness of lysozyme in food applications (Gill and Holley, 2002; Davidson and Harrison, 2002). The antimicrobial effect of lysozyme, particularly against Gram-negative bacteria, can often be enhanced by the presence of specific chemicals or physical treatments (Table 12.3). Sensitivity of Gram-negative species to lysozyme was increased by chelators, certain antibiotics, amino acids, alkaline pH, osmotic shock, drying, and freeze thawing (Tranter, 1994; Proctor and Cunningham, 1988) and by hydrogen peroxide and ascorbic acid (Miller, 1969). Lysozyme activity was also enhanced by food additives including EDTA, butyl paraben, and tripolyphosphate, as well as naturally occurring antimicrobials such as conalbumin, lactoferrin, nisin, and transferrin (Johnson, 1994). Other food components promoting lysozyme activity include lactic acid, trypsin, proteinase K, and lipase (Sofos et al., 1998).

Many chemical agents can permeabilize the outer membrane of Gram-negative bacteria, as classically shown by the metal chelator EDTA. Early studies demonstrated that Versene (EDTA) could increase the lytic activity of lysozyme against certain Gram-negative bacteria (Repaske,

TABLE 12.3
Physical Treatments and Chemical Agents Reported to
Increase the Activity of Lysozyme against Selected
Organisms

Physical Treatments
Heat
Alkaline pH
Osmotic shock
Freeze thawing
High pressure
Chemical Treatments
Ethylenediamine tetraacetic acid (EDTA), diethylene triamine pentaacetate (DTPA), other divalent metal chelators
Trisodium phosphate
Cysteine, proline
Conalbumin
Lactoferrin, other transferrins
Lysolecithin, polyethylene glycol, other surfactants
Glycine
Poly-L-lysine
Nisin, possibly other bacteriocins
Hydrogen peroxide
Ascorbic acid
Lactic acid
Trypsin, proteinase K
Lipase
Carvacrol, nerolidol, certain other terpenoids

1958). Chelators such as EDTA may remove stabilizing divalent cations from lipopolysaccharide in the outer membrane, increasing permeability of the cells to antimicrobial agents (Hancock, 1984).

As determined by a spiral gradient end point test, lysozyme (up to 4760 µg/ml) in media inhibited growth of only one (*Lactobacillus lactis*) of seven Gram-positives and none of eight Gram-negative foodborne bacteria tested, but addition of EDTA resulted in inhibition of all Gram-positive (*Lactobacillus lactis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and four of eight Gram-negative strains (*Yersinia enterocolitica*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, and *Pseudomonas putida* but not *Enterobacter aerogenes*, *E. coli* O157:H7, *Pseudomonas aeruginosa*, or *Salmonella* Typhimurium) (Razavi-Rohani and Griffiths, 1996a). The combination EDTA–Tris–lysozyme was bactericidal to eight of ten Gram-negative bacterial pathogens tested in buffer and two of six Gram-positive bacteria tested, including *L. monocytogenes* (Wooley and Blue, 1974). The presence of EDTA increased the activity of lysozyme against *C. botulinum* and *L. monocytogenes* (Hughey and Johnson, 1987) as well as *Bacillus subtilis* and *Pseudomonas aeruginosa* (Vakil et al., 1969). Lysis of coliforms implicated in bovine mastitis by bovine sera was markedly increased on addition of lysozyme and EDTA, although lysozyme alone did not lyse the cells (Carroll, 1979). Growth of predominantly Gram-negative shrimp microflora in nutrient broth was slightly delayed by 150 µg/ml lysozyme but strongly inhibited by 50 µg/ml lysozyme in the presence of 0.02% EDTA (Chander and Lewis, 1980). Lysozyme alone was shown to inhibit only two of twelve fungal species tested, although all but one species were inhibited by a lysozyme–EDTA combination (Razavi-Rohani and Griffiths, 1999).

The potentiation of lysozyme activity by EDTA has also been demonstrated in food systems. Although lysozyme alone was not effective against *Salmonella* Typhimurium in culture medium, both lysozyme and a lysozyme–EDTA combination (1 or 5 mg/ml, respectively) applied for 180 minutes in a dip system significantly reduced numbers of the organism on inoculated broiler legs (Samuelson et al., 1985). EDTA (1 to 5 mM) increased the antimicrobial activity of egg-white lysozyme against *L. monocytogenes* in several foods (Hughey et al., 1989). Lysozyme (3 mg/ml) in combination with EDTA (5 to 25 mM) on cod fish was more effective against *L. monocytogenes* than lysozyme alone (Wang and Shelef, 1992). In ultra-high temperature (UHT) milk, lysozyme in combination with EDTA was bactericidal to *L. monocytogenes* and slowed growth of *E. coli* O157:H7 (Payne et al., 1994). However, growth of *Pseudomonas fluorescens* and *Salmonella* Typhimurium in UHT milk was not affected by lysozyme, even in combination with EDTA (Payne et al., 1994). The use of EDTA and certain other chelators in foods would not be beneficial in many food systems because it could decrease the nutritional value by binding needed minerals.

The effectiveness of lysozyme against *C. botulinum* can be increased using chelators such as EDTA, diethylene triamine pentaacetate (DTPC), and cysteine (Johnson, 1994). EDTA, nisin, butylparaben, and tripolyphosphate increased the delay of botulinum toxin production by lysozyme in bratwurst, salmon, and cod suspensions (Johnson, 1994). Toxin production by *C. botulinum* was also delayed in turkey suspensions, potatoes, asparagus, and salmon by the addition of lysozyme, and the delay was increased in the presence of EDTA (Johnson and Dell'Acqua, 1995).

Combinations of lysozyme and nisin, a food preservative effective against Gram-positive bacteria, are effective against numerous pathogenic and spoilage bacteria. Because lysozyme is generally less expensive than nisin, lysozyme–nisin combinations may be cost-effective alternatives to the use of nisin alone for the control of Gram-positive organisms in some foods. Nisin concentrations of 100 to 500 units/g are usually sufficient to increase the antimicrobial effectiveness of lysozyme (Tranter, 1994). The increased efficacy of lysozyme–nisin combinations may be the result of increased membrane damage, cell lysis, or the inhibition of energy-dependent processes that repair lysozyme–nisin damage to the cell (Chung and Hancock, 2000). The presence of lysozyme increased the depolarization of the cytoplasmic membranes of *S. aureus* by nisin (Chung and Hancock, 2000). Severe cell damage in the form of surface disruption was observed by scanning electron microscopy in *Lactobacillus sake* treated with lysozyme–nisin combinations (Chung and Hancock, 2000).

Lysozyme and nisin in combination were more effective than lysozyme alone against many lactic acid bacteria in culture media (Chung and Hancock, 2000), and can be bacteriostatic or bactericidal to *L. monocytogenes* under certain conditions (Monticello, 1990). Growth of *L. monocytogenes* was inhibited on sliced pork bologna by the use of a dip containing nisin and lysozyme, although lysozyme alone had no effect (Kain et al., 2001). Lysozyme–nisin combinations were generally more effective than either lysozyme or nisin alone to control the growth of the Gram-positive meat spoilage bacteria *Brochothrix thermosphacta* and *Carnobacterium* species in culture media, in meat juice extract, and on pork tissue (Nattress et al., 2001). Addition of 500 mg/kg lysozyme–nisin (1:3 mixture) and 500 mg/kg EDTA to ham or bologna sausage batter before cooking resulted in growth inhibition at 8°C of *Brochothrix thermosphacta*, *Listeria monocytogenes*, *Lactobacillus curvatus*, *Lactobacillus mesenteroides* on sausage and of *E. coli* O157:H7, *Lactobacillus mesenteroides*, *Brochothrix thermosphacta*, and *Lactobacillus curvatus* on ham (Gill and Holley, 2000a). The same microbial combination was ineffective against *L. sake*, *Salmonella* Typhimurium, *Serratia grimesii*, and *Shewanella putrefaciens* on both products (Gill and Holley, 2000a). Combinations of lysozyme, nisin, and hop acids were bactericidal to *L. monocytogenes*, *B. cereus*, and *Bacillus subtilis* in culture media and to *L. monocytogenes* on hot dogs or cooked hams when applied as a dip or spray, respectively (King and Ming, 2002). However, without nisin, hop resins did not increase the effectiveness of lysozyme against *E. coli* (Fukao et al., 2000). When added as a third preservative factor, lysozyme increased the bactericidal activity of nisin and

carvacrol against *B. cereus* and *L. monocytogenes* in buffer (Pol and Smid, 1999). The bactericidal activity of pulsed electric field (PEF) against *Salmonella* Typhimurium in orange juice was increased in the presence of lysozyme or a combination of lysozyme and nisin (Liang et al., 2002).

Although nisin–lysozyme combinations are effective against many Gram-positive bacteria, lactoferrin may enhance the effectiveness of lysozyme against Gram-negative bacteria. Lactoferrin, a protein found in milk, appears to damage the outer membrane of Gram-negative bacteria, causing the release of LPS molecules and increasing bacterial susceptibility to lysozyme by increasing penetration of lysozyme through the outer membrane (Ellison and Giehl, 1991; Yamauchi et al., 1993). Lactoferrin has been shown to enhance the antimicrobial activity of lysozyme against *E. coli* (Yamauchi et al., 1993, Suzuki et al., 1989). The combination of apo-lactoferrin and lysozyme delayed growth of *L. monocytogenes* in UHT milk but was not as effective as against the organism as an EDTA–lysozyme combination (Payne et al., 1994). Egg-white lysozyme (0.08 mg/ml) increased the antimicrobial effect of bovine lactoferrin against *Salmonella* Enteritidis in buffer, although lysozyme alone was not bactericidal to the organism (Facon and Skura, 1996). Human lysozyme and human milk lactoferrin were each bacteriostatic to *Vibrio cholerae*, *Salmonella* Typhimurium, and *E. coli* in culture media but were often bactericidal to all three organisms when used in combination under certain conditions (Ellison and Giehl, 1991). The bactericidal effect of lysozyme–lactoferrin combinations is dose-dependent, is blocked by iron saturation of lactoferrin, and is inhibited by high calcium levels (Ellison and Giehl, 1991).

Numerous other substances commonly found in foods have been shown to affect lysozyme activity. Glycine–lysozyme combinations are effective against Gram-positive and Gram-negative bacteria and are used commercially in Japan in certain foods (Proctor and Cunningham, 1988). Lysozyme at 100 µg/g delayed botulinum toxin production by in aqueous turkey slurries, especially in the presence of cysteine, proline, and sodium lactate (Johnson, 1994). *Pseudomonas aeruginosa*, but not *E. coli* O157:H7 or *Salmonella* Typhimurium, was sensitized to lysozyme by lactic acid or HCl disruption of the outer membrane (Alakomi et al., 2000). Lysis of *Actinomyces*, *Pediococcus*, *Propionibacterium*, and *Lactobacillus* strains was demonstrated using lysozyme suspended in buffer containing polyethylene glycol (Chassy and Guiffrida, 1980).

Purified lipase, but not polyphosphates, increased the bactericidal activity of lysozyme against *L. monocytogenes* in buffer (Liberti et al., 1996). However, trisodium phosphate increased the susceptibility of *Campylobacter jejuni*, *E. coli*, *Pseudomonas fluorescens*, *Salmonella* Enteritidis, and *L. monocytogenes* to lysozyme both in water and, to a lesser extent, on chicken skin (Carneiro del Melo et al., 1998). Sublethal levels of trisodium phosphate may cause disruption of the outer membrane of Gram-negative bacteria (Carneiro del Melo et al., 1998).

Lysozyme activity can be increased by physical processes used in many food preservation systems. Freezing and thawing of *E. coli* causes sensitivity to lysozyme (Kohn, 1960; Ray et al., 1984), although the organism regained resistance after incubation at 37°C or exposure to calcium (Ray et al., 1984). Transmission electron microscopy demonstrated protoplast formation and cell-wall damage in unfrozen or frozen/thawed *L. monocytogenes* treated with lysozyme (El-Kest and Marth, 1992).

The sensitization of Gram-negative cells to lysozyme by high pressure is dependent on factors such as pressure, temperature, pH, medium, growth stage, and bacterial species (Masschalck et al., 2001). High pressure has been shown to sensitize *E. coli* to lysozyme (Garcia-Graells et al., 1999; Masschalck et al., 2000), although the sensitization is transient (Hauben et al., 1996; Masschalck et al., 2001). In addition, four of six Gram-negative bacteria tested were sensitive to lysozyme during treatment by high hydrostatic pressure (Masschalck et al., 2001). EDTA increased the activity of lysozyme against *E. coli* during high pressure treatment (Hauben et al., 1996). However, exposure of *E. coli* to lysozyme or lysozyme–EDTA immediately after high-pressure treatment did not decrease the number of viable cells (Hauben et al., 1996). Pulses of high hydrostatic pressure may sensitize some bacterial strains more effectively to lysozyme than treatment with continuous pressure (Masschalck et al., 2001). Sublethal injury by high pressure might be a useful hurdle along

with lysozyme or lysozyme–EDTA in certain foods. Lower pressures required to inactivate certain bacteria in the presence of lysozyme (Masschalck et al., 2001) may lead to new combination treatments for the nonthermal preservation of foods.

FOOD APPLICATIONS OF LYSOZYME

Egg-white lysozyme is desirable as a food preservative because of its ease of purification in economically feasible quantities from egg white, low toxicity, specific activity against target bacteria and fungi, low effective usage levels, and low impact on sensory qualities of foods (Sofos et al., 1998). Lysozyme is stable under many conditions typically found in foods and is highly soluble in aqueous environments (Razavi-Rohani and Griffiths, 1996b). Lysozyme usually is used in foods at 20 to 400 ppm (Gould, 2002), and commercial preparations for food use are generally available in powdered or granular form. Possible limitations of lysozyme include cost, inactivation by endogenous food components, and limited antimicrobial spectrum (Holzapfel et al., 1995).

As described earlier in this chapter, lysozyme is currently used in foods such as cheese, frankfurters, cooked meat, and poultry products (Davidson and Harrison, 2002). A patented process in the United Kingdom applies to the addition of lysozyme to butter or milk used to make Italian cheeses so as to control undesirable organisms such as *C. tyrobutyricum* (Ferrari and Dell'Acqua, 1979). Lysozyme, used in combination with standard preservatives, reduced microbial spoilage of cooked, salami, and Vienna sausages (Akashi, 1969, 1970, 1971). Microbial lytic enzymes may also have applications as food preservatives. An N-acetylmuramidase purified from *Streptomyces rutgerensis* extended the shelf life of adzuki bean paste (Hayashi et al., 1989).

Lysozyme is commonly used instead of nitrate to prevent late blowing in hard and semi-hard cheeses such as Gouda, Edam, provolone, and Emmentaler, in the United States and numerous other countries (International Dairy Federation, 1987). Late blowing is a ripening defect resulting from hydrogen and carbon dioxide gas production during the fermentation of lactate by *C. tyrobutyricum*, a heat-resistant spore former commonly found in milk. Lysozyme is usually added to milk at 20 to 35 ppm, resulting in levels in cheese ranging from 200 to 400 ppm, which does not generally inhibit starter cultures (Sofos et al., 1998) or affect physical or organoleptic properties of the cheese. Approximately 100 tons of lysozyme are used annually to prevent the blowing of cheese (Scott et al., 1987).

Numerous Japanese patents cover the use of lysozyme as a preservative in foods such as fresh vegetables, meats, seafood such as oysters and shrimp, tofu, cheeses, butter, potato salad, noodles, custard, and infant milk formula (Tranter, 1994; Cunningham et al., 1991). Lysozyme has been investigated extensively as a preservative in vegetable, meat, and seafood products in Japan (Tranter, 1994) and has shown effectiveness as a preservative in products such as kimchi (fermented cabbage), rice sushi, noodles, and creamed custard (Cunningham et al., 1991). Other Japanese patents apply to use of lysozyme applied as a coating on fruits, vegetables, meats, and seafoods (Cunningham et al., 1991; Sofos et al., 1998). Lysozyme used in a gelatin dip extended the shelf life of a type of seafood product made from lizard-fish (Akashi and Oono, 1972).

Lysozyme has been used in Japan to extend the shelf life of wine and sake (Sofos et al., 1998, Tranter, 1994) and is used with increasing frequency to improve the quality of wines produced in a number of other countries. The addition of 250 mg/L lysozyme after malolactic fermentation increased the microbiological stability of red wine (Gerbaux et al., 1997). Such inhibition of spoilage bacteria in wine by lysozyme may contribute to use of lower sulfite levels in some products.

Numerous patents have been issued in the United States for lysozyme applications in food systems. These include patents for listericidal treatments using lysozyme in dairy or meat products (Dell'Acqua et al., 1989); lysozyme and EDTA on vegetables (Johnson et al., 1991); and combinations of hop acids, nisin, and/or lysozyme as ingredients in or applied to the surface of solid foods (King and Ming, 2002). Another patent covers the use of lysozyme or nontoxic lysozyme salts along with chelators such as EDTA to control growth of *Clostridium botulinum* in animal or

vegetable foods (Johnson and Dell'Acqua, 1995). Lysozyme added to infant formula or milk encourages the growth of *Lactobacillus* species (Schwimmer, 1981) and *Bifidobacterium bifidus* (Sawada et al., 1967; Nishihava and Isoda, 1967) in infant intestines, which may prevent growth of harmful bacteria.

Lysozyme may also be effective in immersion or spray methods to decontaminate animal carcasses. Gram-negative organisms, including *Salmonella*, can become sensitive to lysozyme after a sudden decrease in osmolarity, possibly increasing the antimicrobial effectiveness of lysozyme used in the decontamination of poultry (Chatzopolou et al., 1993).

Combinations of lysozyme with other antimicrobials may be useful when applied to the surface of meat products, either through a dip, by application of an edible gel, or on packaging film (Gill and Holley, 2000a). Use of lysozyme–antimicrobial combinations as a surface application in packaging components would prevent inactivation of the enzyme during cooking (Gill and Holley, 2000b). A lysozyme–nisin gel coating (1:3 mixture; 25.5 mg/ml) with 25.5 mg/ml EDTA applied to the surface of cooked ham and bologna was bactericidal to *Brochothrix thermosphacta*, *L. sakei*, *Leuconostoc mesenteroides*, *L. monocytogenes*, and *Salmonella* Typhimurium and inhibited growth of the organisms during storage at 8°C for 4 weeks (Gill and Holley, 2000b). Nisin and lysozyme incorporated together into whey-protein isolate film was antimicrobial to *Salmonella* Typhimurium colonies on agar plates (Rodrigues et al., 2002). Lysozyme incorporated into biodegradable packaging films was bactericidal to *Lactobacillus plantarum* and, in combination with EDTA, *E. coli* (Padgett et al., 1998). Incorporation of lysozyme into packaging films would allow direct contact of antimicrobials with pathogens present on the surface of foods.

The use of lysozyme on food contact surfaces may prevent colonization by pathogens such as *L. monocytogenes* as well as other biofilm-forming bacteria (Bower et al., 1998). Hen egg-white lysozyme forms multilayers on hydrophobic metal surfaces (Schmidt et al., 1990). However, some loss of antimicrobial activity by adsorbed lysozyme has been demonstrated (Schmidt et al., 1990). Lysozyme treatment of stainless steel resulted in the attachment of increased numbers of vegetative *Bacillus* species, possibly as a result of removal of extracellular polysaccharides by the enzyme (Parker et al., 2001).

CHEMICALLY MODIFIED LYSOZYME MOLECULES AS ANTIMICROBIALS

Lysozyme–macromolecule conjugates have shown increased antimicrobial activity. Attachments of fatty acids to lysozyme, which occurred without loss of enzymatic activity, resulted in increased bactericidal activity against Gram-negative bacteria, an effect attributed to enhanced membrane-fusion capabilities (Ibrahim et al., 1991; Ibrahim et al., 1993; Ibrahim et al., 2002; Liu et al., 2000). Conjunction of lysozyme with perillaldehyde increased the antimicrobial activity against *E. coli* and *S. aureus* (Ibrahim et al., 1994a). Similarly, a lysozyme–dextran conjugate was antimicrobial to Gram-positive and Gram-negative bacteria and also had excellent emulsifying properties (Nakamura et al., 1990; Nakamura et al., 1991). A lysozyme–galactomannan conjugate was bactericidal to Gram-negative bacteria and had increased emulsifying properties (Nakamura et al., 1992). Galactomannan, or guar gum, is commonly used in foods as a thickener, binder, or stabilizer. Increased emulsifying properties of some conjugates may allow lysozyme penetration through the outer membrane, allowing access to the peptidoglycan substrate. Lysozyme–polysaccharide conjugates may be suitable for use in foods. Other modifications of lysozyme have increased the effectiveness of lysozyme against Gram-negative organisms. For instance, modified lysozyme with a hydrophobic pentapeptide at its C-terminus displayed greater action against *E. coli* than wild-type egg-white lysozyme (Ibrahim et al., 1994b). Chemically derivatized molecules of lysozyme appear to have much potential for antimicrobial applications, but the mechanisms and food and clinical utility will require more research, food challenge studies, and clinical trials.

NONENZYMATIC ANTIMICROBIAL ACTIVITY OF LYSOZYME AND LYSOZYME PEPTIDES

The antimicrobial activity of lysozyme resulting from its enzymatic muramidase activity on bacterial cell walls has been studied extensively. However, lysozyme has also been reported to have antimicrobial activity unrelated to its enzymatic activity (Chung and Hancock, 2000).

Cationic proteins often possess bactericidal properties (Pelligrini et al., 1992). The basic nature of the lysozyme molecule itself may result in antimicrobial activity against some organisms (Mayes and Takeballi, 1983; Salton, 1957). For instance, *S. aureus* will grow in egg white at pH 7.2 but not at pH 8.0, possibly because of negative charges on the cell leading to agglutination by positively charged molecules such as lysozyme (Ng and Garibaldi, 1975). Treatment of *E. coli* with egg-white lysozyme resulted in disintegration of the cell cytoplasm visible by electron microscopy, and lysozyme was detected within the cytoplasm of frozen cells by immunogold labeling (Pelligrini et al., 1992).

Bactericidal properties of denatured or partially denatured lysozyme against Gram-negative and Gram-positive bacteria have been shown, with increased sensitivity of *E. coli* to heat-denatured lysozyme compared to native lysozyme (Ibrahim et al., 1996a, 1996b). Pelligrini and others (1992) found that denaturation of lysozyme by dithiothreitol (DTT) to reduce disulfide bonds did not lead to a loss of antimicrobial activity against Gram-negative and Gram-positive bacteria, suggesting that the bactericidal efficacy of lysozyme is the result not only of lytic muramidase activity but also of cationic and hydrophobic properties (Pelligrini et al., 1992). Native lysozyme and lysozyme denatured by DTT were equally bactericidal to oral streptococci (Liable and Germaine, 1985). The microbiocidal activity of T4 lysozyme may be the result of membrane disruption by small peptide sequences rather than enzymatic cell-wall degradation (During et al., 1999). Heat-denatured bacteriophage T4 lysozyme exhibited antimicrobial activity but no enzymatic activity (During et al., 1999).

Despite these intriguing results, enzymatic activity is still considered by some researchers to be necessary for full antimicrobial activity of native lysozyme and heat-denatured lysozyme and for the bactericidal effect of lysozymes under pressure (Masschalck et al., 2001). Egg-white lysozyme, partially denatured lysozyme, denatured lysozyme, and synthetic cationic peptides from lysozymes were not bactericidal or bacteriostatic to Gram-negative bacteria at atmospheric pressure in one study (Masschalck et al., 2001). Four of six Gram-negative bacteria tested were sensitive to native lysozyme during treatment by high hydrostatic pressure (Masschalck et al., 2001). Heat denaturation to inactivate muramidase activity eliminated the bactericidal effect of native lysozyme against Gram-negative bacteria under pressure, although partially denatured lysozyme was still active (Masschalck et al., 2001).

Antimicrobial cationic peptides with no enzymatic activity have been isolated from lysozyme (Pelligrini et al., 1997; During et al., 1999). Peptide digests of lysozyme without enzyme activity were strongly bactericidal to *E. coli* and *S. aureus*, with damage to cell membranes of both species evident by scanning electron microscopy (Mine et al., 2002). A 15-amino-acid sequence (amino acids 98–112) isolated from egg-white lysozyme and an identical synthesized peptide both exhibited antimicrobial activity without catalytic muramidase activity (Pelligrini et al., 1997). However, the peptide sequences were weaker against Gram-negative bacteria than parent lysozyme and were ineffective against some organisms that were lysed by the parent lysozyme, such as *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *S. aureus* (Pelligrini et al., 1997). Synthetic peptides similar to C-terminal areas on T4 lysozyme were also microbiocidal, causing cell membrane damage to bacterial, fungal, and plant cells (During et al., 1999). Synthetic cationic peptides of egg-white and T4 lysozyme also sensitized Gram-negative bacteria to high pressure (Masschalck et al., 2001). In the future, protein engineering may lead to the optimization of lysozyme peptide fragments as antimicrobial agents (During et al., 1999).

RECOMBINANT LYSOZYMES FOR USE AS ANTIMICROBIALS IN FOODS

For several years, researchers have been interested in expressing recombinant (cloned) human lysozyme, egg-white lysozyme, or lysozymes from other sources. Recombinant lysozyme from humans or other sources may have reduced allergenicity compared with egg-white lysozyme. Directed mutations of the cloned lysozyme gene could result in lysozymes with enhanced resistance to intrinsic and extrinsic factors in foods, increased specificity for target molecules, and other desirable features. Lysozyme from human, bovine, and microbial sources has been expressed in organisms that are often compatible for use in foods (Sofos et al., 1998). For instance, active egg-white lysozyme was expressed by *Saccharomyces cerevisiae* (Oberto and Davison, 1985) and *Aspergillus niger* (Archer et al., 1990). Inactive egg-white lysozyme but active T4 and bacteriophage lysozymes were produced by transformed *Lactococcus lactis* subspecies *lactis* strains (van de Guchte et al., 1992). Human lysozyme has been expressed during growth in whey by the yeast *Kluyveromyces lactis* after transformation with the human lysozyme, potentially leading to use of the yeast as a lysozyme-producing starter culture in cheeses prone to late blowing caused by clostridial fermentation (Maullu et al., 1999). Similarly, human lysozyme secreted into the milk of transgenic mice retained its lytic activity, leading to potential applications in the dairy industry of lysozyme-containing milk produced by transgenic animals (Maga et al., 1995; Maga et al., 1998). Transgenic potato plants expressing T4 lysozyme have also been developed (During et al., 1999).

Genetic engineering resulted in a modified T4 lysozyme with a new disulfide bond, which increased thermostability by stabilizing the tertiary structure (Genentech and Genencor, 1985). Such modifications may increase applications of lysozyme in cheesemaking and other food applications (Proctor and Cunningham, 1988). A hyperstable chicken lysozyme was isolated by directed mutations and expression (Shih and Kirsch, 1995). Increased numbers of recombinant enzymes with advantages over native are being introduced into the food industry, but their safety must be carefully evaluated prior to their use in commerce (Pariza and Johnson, 2001).

REGULATORY STATUS AND TOXICOLOGY

Egg whites are well known to be allergenic to sensitive individuals, particularly children (Poulsen et al., 2001), and lysozyme may constitute one of the allergens in egg albumin. However, the most allergenic proteins in eggs appear to be (in descending order) conalbumin (ovotransferrin), ovomucoid, ovalbumin, and lysozyme. Allergic reactions produced by egg-white lysozyme in animals and humans were less than reactions to other egg proteins such as ovalbumin and albumin (Bianchi, 1982), which have long been used as food ingredients. The acute toxicity of lysozyme in humans is unknown (Lück and Jager, 1997), although rodents and rabbits tolerated high intravenous doses (Barbara and Pellegrini, 1976; Bianchi, 1982). Several studies have indicated that lysozyme from egg white has negligible acute, subacute, and chronic toxicity in animals (Barbara and Pellegrini, 1976). In addition, lysozyme has a long history of safe consumption as an endogenous food ingredient in eggs, milk, and other foods. The presence of added egg-white lysozyme in foods at permitted levels is generally not considered to be a health concern.

Lysozyme is considered as generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) for use in certain cheeses (U.S. FDA, 1998). However, because of the potential for allergenicity of lysozyme, the FDA has tentatively concluded that bulk and packaged foods containing lysozyme must be labeled to indicate presence of "egg-white lysozyme" (U.S. FDA, 1998). This is a condition of lysozyme use for GRAS status in such treated cheese. It is permitted for use in cheese at levels in accordance with good manufacturing practices. In addition, the Joint Food and Agriculture Organization/World Health Organization concluded that the low additional intake of lysozyme in cheese made from treated milk was not a hazard to consumer health (JECFA, 1993). Use of hen egg-white lysozyme has been approved in many countries to control the growth of spoilage organisms in foods (Wang and Shelef, 1992). Lysozyme was approved

as a preservative (E1105) under the European Additives Directive to prevent late blowing in ripened cheese. Lysozyme is also approved for use in cheese in Austria, Australia, Belgium, Canada, Denmark, Finland, France, Germany, Italy, the United Kingdom, and Spain. The Organisation Internationale du Vin (OIV) approved the use of lysozyme in wine in 1997 to control undesirable bacterial growth, and its use in wines is awaiting approval in other countries. Applications for uses in other foods are being evaluated. The United States Department of Agriculture in 2002 issued an acceptability determination regarding the use of a mixture of hops beta acids, egg-white lysozyme, and cultured skim milk in salad dressings used as a preservative in refrigerated meat and poultry deli salads (USDA, 2002). At the time of this chapter preparation, the FDA has not yet made a final GRAS ruling regarding the use of egg-white lysozyme for use as an antimicrobial agent in casings for frankfurters and on ready-to-eat cooked meat and poultry products (U.S. FDA, 2001).

SUMMARY AND PERSPECTIVES

In response to increased consumer perception and demand for more nutritious foods, the food industry is introducing foods into the marketplace that receive minimal processing and contain reduced levels of traditional preservatives, such as salt, sugar, and acids, and few or no chemical preservatives (Johnson, 1994; Sofos et al., 1998). To attain adequate shelf life for quality and to prevent microbial foodborne disease, there is significant interest by the industry in the use of natural preservation systems. A myriad of naturally occurring antimicrobials are being considered to develop safety systems usually based on a multiple hurdle system to inactivate or prevent the growth of pathogenic or spoilage organisms in foods (Sofos et al., 1998). The combination of more than one “hurdle” often results in increased microbial stability of foods (Scott, 1989). In designing these systems, it is important to consider the interaction and multiple functions of antimicrobials, as pioneered in the writings of Reiter (1978) and others. Naturally occurring antimicrobials, in conjunction with good manufacturing practices, can enhance the safety and quality of minimally processed refrigerated foods (Johnson, 1994). Egg-white lysozyme is a good example of a naturally occurring enzyme that has been used by the industry to maintain product quality and reduce the incidence of spoilage.

Combinations of natural antimicrobials and mild preservation techniques are attractive approaches to control pathogens and spoilage organisms in many foods. The addition of lysozyme to certain foods before heat processing may reduce the thermal requirements necessary to inactivate spores of some strains of thermophilic sporeforming spoilage bacteria that are particularly sensitive to lysozyme, such as *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* (Johnson, 1994).

The antimicrobial effectiveness of lysozyme and its commercial application as a food preservative needs to be further investigated, especially in refrigerated foods under conditions similar to actual processing and storage of products. Salt levels for optimum lysozyme activity need to be determined for individual food systems. Additional research is needed to verify that lysozyme does not interfere with growth of beneficial bacterial and yeast cultures in fermented foods. More research is also needed to develop optimal systems comprised of combinations of antimicrobials for maximal efficacy.

Lysozyme could play a key role in the development of antimicrobial systems for use in foods. Lysozyme is relatively inexpensive, is readily available, and is stable under a wide variety of conditions. Lysozyme and lysozyme–antimicrobial combinations have many current and potential uses in the food industry to control pathogenic and spoilage organisms. Activity of lysozyme occurs over a wide range of temperatures and pHs. It is among the most stable of enzymes in foods and resists many food processing procedures. These properties may lead to numerous applications for lysozyme minimally processed foods. The use of lysozyme in these foods for control of pathogens and spoilage organisms may result in products with increased safety, enhanced quality, and long shelf life.

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13 Bacteriocins with Potential for Use in Foods

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CONTENTS

Classification of Bacteriocins	390
Genetics, Biosynthesis, and Mode of Action	391
Organization of Gene Clusters	391
Biosynthetic Pathway.....	392
Posttranslational Modification, Activation, and Transport.....	393
Regulation of Biosynthesis	395
Producer Immunity	395
Mode of Action.....	396
Determination of Bacteriocin Activity	397
Agar Plates	397
Newer Methods.....	398
Production and Modeling.....	398
Resistance.....	399
Examples of Activity Spectra and Biochemical Properties	400
<i>Lactococcus</i>	400
<i>Pediococcus</i>	402
<i>Lactobacillus</i>	406
<i>Carnobacterium</i>	410
<i>Leuconostoc</i> and Other Lactic Acid-Associated Bacteria.....	411
Bacteriocins in Packaging Film.....	414
Closing Remarks	415
References	416

Interest in the development of bacteriocins as food preservatives and antimicrobial agents has robustly continued into the 21st century. A substantial portion of this effort has occurred in food technology laboratories working with bacteriocins produced by lactic acid bacteria (LAB). In the United States, the authorization by the U.S. Food and Drug Administration (FDA) in 1988 for use of nisin as a food additive in pasteurized processed cheese spreads opened the door for the potential application of other bacteriocins as preservatives in foods. Covered earlier in this book (Chapter 7), nisin is an antibiotic-like, heat-stable peptide produced by *Lactococcus lactis* subspecies *lactis*, which, among bacteriocins produced by LAB, is the most potent bacteriocin known. It is effective against an array of Gram-positive bacteria, most notably vegetative types and spores that are involved in foodborne illness and food spoilage. The effectiveness of nisin as a processing aid has been demonstrated in many products worldwide for more than 50 years. An important contributory factor for the sustained interest in bacteriocins is the great concern that has grown in the food

industry regarding *Listeria monocytogenes*, especially in American processed foods where there is zero tolerance for the pathogen. *L. monocytogenes* is a Gram-positive, psychrotrophic bacterium that is relatively common in raw foods and stubbornly resists normal means of control and elimination. Its human foodborne syndrome can sometimes be fatal, and the infective dose is highly variable among human subpopulations. The relevance is that nisin and other bacteriocins produced by some strains of LAB are specifically antagonistic against *L. monocytogenes*. Therefore, with the legal precedent set for use of nisin as an additive in foods in the United States, the potential exists for application of other bacteriocins as effective inhibitive agents against *L. monocytogenes* and other important Gram-positive pathogens to enhance product safety. The production of bacteriocins from lactic cultures potentially shortens the regulatory process because most LAB have a GRAS (generally regarded as safe) status, having been consumed in large numbers in acidified fermented foods by people for thousands of years with no ill effects. In this regard, bacteriocins from LAB have been described as “natural” inhibitors. This chapter focuses on review of bacteriocins from LAB exclusive of nisin. Using current taxonomy, LAB associated with foods include members of the genera *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Stiles and Holzappel, 1997). We have not attempted to inclusively present the vast volume of information that now exists in this area. We have primarily addressed bacteriocins from the first six LAB genera listed previously with inclusion of some specific examples of work on bacteriocins from *Propionibacterium* and *Bifidobacterium*, given the importance of these two genera in foods.

CLASSIFICATION OF BACTERIOCINS

The prototype bacteriocins were the colicins. First discovered by Gratia in 1925, “principe V” was produced by one strain of *Escherichia coli* against another culture of *E. coli*. The term “colicine” was coined by Gratia and Fredericq (1946); “bacteriocine” was used by Jacob et al. (1953) as a general term for highly specific antibacterial proteins. The term colicin now implies a bacteriocidal protein produced by varieties of *E. coli* and closely related Enterobacteriaceae (Konisky, 1982).

The original meaning of the term bacteriocin was thus greatly influenced by characteristics common to colicins — that is, bacteriocins were originally defined as bacteriocidal proteins characterized by lethal biosynthesis, a very narrow range of activity, and adsorption to specific cell envelope receptors (Jacob et al., 1953). A later amendment was the association of bacteriocin biosynthesis with plasmids. The description has expanded to recognize the differences between colicins and bacteriocins produced by Gram-positive bacteria (Tagg et al., 1976). Bacteriocins from Gram-positive bacteria usually do not possess a specific receptor for adsorption; are commonly of lower molecular weight than colicins; can have different modes of killing, a wider range of effect, and different modes of release and cell transport; and can possess leader sequences cleaved during maturation (Jack et al., 1995; James et al., 1991; Riley, 1998). Today, bacteriocidal peptides or proteins produced by bacteria are labeled as bacteriocins. Normally, to demonstrate the proteaceous nature of a newly characterized bacteriocin, sensitivity to proteolytic enzymes such as trypsin, α -chymotrypsin, and pepsin is an expected demonstration. Usage in food preservation usually requires estimation of its heat resistance given the widespread use of thermal processing in food production.

Bacteriocins are widely found throughout the bacterial world. These compounds are assumed to provide producing bacterial cells with a selective advantage over other bacteria. Synthesis of bacteriocins is found across all major groups of eubacteria and archaebacteria (Riley, 1998). There have been many valuable monographs written over the years that review colicins, bacteriocins, bacteriocins from LAB, and applications of specific bacteriocins (Reeves, 1972; Franklin and Snow, 1975; Hardy, 1975; Tagg et al., 1976; Konisky, 1982; Klaenhammer, 1988, 1993; Jack et al., 1995; de Vos et al., 1995; Sahl et al., 1995; Venema et al., 1995a,b,c; Abee et al., 1995; Nes et al., 1996; Cleveland et al., 2001).

Normally, bacteriocins from LAB are cationic and are hydrophobic or amphiphilic molecules composed of 20 to 60 amino acid residues (Nes and Holo, 2000). Bacteriocins produced by Gram-positive bacteria are commonly classified into three classes (Klaenhammer, 1993; Nes et al., 1996).

Class I contains bacteriocins termed lantibiotics (from *lanthionine-containing antibiotic*). They are small (<5 kDa) peptides containing the unusual amino acids lanthionine (Lan), α -methyl-lanthionine (MeLan), dehydroalanine, and dehydrobutyrine. Class I is further subdivided into Type A and Type B lantibiotics based on their chemical structures and their antimicrobial activities (Moll et al., 1999; van Kraaij et al., 1999; Guder et al., 2000). Type A lantibiotics are elongated peptides with a net positive charge. They exert their activity through the formation of pores in bacterial membranes. Some examples of type A lantibiotics are nisin (Hurst, 1981), lactocin S produced by *Lactobacillus sake* (Mortvedt et al., 1991), epidermin produced by *Staphylococcus epidermidis* and *Streptococcus mutans* (Allgaier et al., 1986), gallidermin from *S. epidermidis* (Kellner et al., 1988), and lactacin 481 from *L. lactis* (Piard et al., 1992). Type B lantibiotics are smaller globular peptides, which have a negative or no net charge. Their antimicrobial activity is related to the inhibition of specific enzymes. Some examples are mersacidin from *Bacillus* subspecies; *cinnamycin* from *Streptomyces cinnamoneus*; *ancovenin* and *duramycin* from *Streptomyces* and *Streptovercillium* species; and *actagardine* from *Actinoplanes liguriae* (Sahl and Bierbaum, 1998; Altena et al., 2000; Guder et al., 2000).

Class II bacteriocins are the largest group that have been characterized. They contain small (<10 kDa) heat-stable, nonlanthionine-containing, unmodified peptides. They can be further divided into three subgroups. Class IIa includes pediocin-like peptides having an N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys. They have attracted much of the attention because of their anti-*Listeria* activity (Ennahar et al., 2000). Some examples are pediocin PA-1/AcH (Henderson et al., 1992; Nieto-Lozano et al., 1992; Motlagh et al., 1992a,b), sakacins A and P (Holck et al., 1992; Tichaczek et al., 1992), leucocin A-UAL 187 (Hastings et al., 1991), mesentericin Y105 (Hechard et al., 1992), enterocin A (Aymerich et al., 1996), divercin V41 (Metivier et al., 1998), and lactococcin MMFII (Ferchichi et al., 2001). Class IIb contains bacteriocins requiring two different peptides for activity. Some examples are lactococcins G and M (van Belkum et al., 1991; Nissen-Meyer et al., 1992); lactacin F (Allison et al., 1994); and plantaricins A, S, EF, and JK (Nissen-Meyer et al., 1993a; Jimenez-Diaz et al., 1995; Anderssen et al., 1998). Class IIc contains the remaining peptides of the class including *sec*-dependent secreted bacteriocins. Some examples are acidocin B (Leer et al., 1995), carnobacteriocin A (Worobo et al., 1994), divergicin A (Worobo et al., 1995), and enterocins P (Cintas et al., 1997) and B (Nes and Holo, 2000).

Class III bacteriocins are large (>30 kDa) heat-labile proteins that are not as well characterized (e.g., helveticins J and V-1829) (Joerger and Klaenhammer, 1986; Vaughan et al., 1992). A fourth class consisting of complex bacteriocins that require carbohydrate or lipid moieties for activity has also been suggested by Klaenhammer (1993); however, bacteriocins in this class have not been characterized adequately at the biochemical level such that the definition of this class still requires additional information (Jimenez-Diaz et al., 1995; McAuliffe et al., 2001).

GENETICS, BIOSYNTHESIS, AND MODE OF ACTION

ORGANIZATION OF GENE CLUSTERS

The genetic determinants involved in the production of class I and II bacteriocins are by far the most studied as a result of the success of nisin and the potential applications of the bacteriocins in these two classes. Bacteriocins are ribosomally synthesized and the genes encoding bacteriocin production and immunity are usually organized in operon clusters (Nes et al., 1996; Sahl and Bierbaum, 1998; McAuliffe et al., 2001). For linear unmodified bacteriocins, which include the plantaricins, carnobacteriocins, and sakacins, it appears that specific inducing peptides or peptide pheromones stimulate synthesis of bacteriocins whose genes are usually located nearby on the same

gene cluster (Quadri et al., 1997; Brurberg et al., 1997; Anderssen et al., 1998). Bacteriocin gene clusters can be located on the chromosome, as in the case of subtilin (Banerjee and Hansen, 1988) and mersacidin (Altena et al., 2000); or plasmids, as in the case of divergicin A (Worobo et al., 1995) and sakacin A (Axelsson and Holck, 1995); or transposons, as in the case of nisin (Rauch and de Vos, 1992) and lacticin 481 (Dufour et al., 2000).

The genetic analysis of the biosynthesis of several lantibiotics, epidermin (Schnell et al., 1992; Bierbaum et al., 1996; Geissler et al., 1996), nisin (Buchmann et al., 1988; Mulders et al., 1991; de Vos et al., 1995), subtilin (Banerjee and Hansen 1988; Klein et al., 1992; Klein et al., 1993; Klein and Entian 1994), lacticin 481 (Piard et al., 1993; Rince et al., 1997; Uguen et al., 2000), and mersacidin (Bierbaum et al., 1995; Altena et al., 2000), has revealed that the genes involved in biosynthesis of lantibiotics generally contain genes coding for the prepeptide (LanA — the abbreviation lan refers to homologous genes of different lantibiotic gene clusters), enzymes responsible for modification reactions (LanB,C/LanM), processing proteases responsible for removal of the leader peptide (LanP), ABC (ATP [adenosine triphosphate]-binding cassette), superfamily transport proteins involved in peptide translocation (LanT), regulatory proteins (LanR, K), and proteins involved in producer self-protection (immunity) (LanI, FEG).

The genetics of many class II bacteriocins such as lactococcins A, B, and M (Holo et al., 1991; van Belkum et al., 1991; Stoddard et al., 1992; van Belkum et al., 1992; Venema et al., 1995b); pediocin PA-1/AcH (Marugg et al., 1992; Motlagh et al., 1992a,b; Bukhtiyarova et al., 1994; Venema et al., 1995a); and plantaricin A (Diep et al., 1994, 1995, 1996) have been studied. Genes encoding the biosynthesis of class II bacteriocins share many similarities in their genetic organizations, consisting of a structural gene coding for precursor peptide, followed immediately by a dedicated immunity gene and genes for a dedicated ABC-transporter and an accessory protein. In some cases, regulatory genes have been found. The accessory proteins are essential for the export of class II bacteriocins. No counterparts of these accessory proteins in lantibiotics have been found (Nes et al., 1996; Sablon et al., 2000). For more elaborate reviews on the genetics of lantibiotics and nonlantibiotics see Klaenhammer (1993), Jack et al. (1995), Nes et al. (1996), Sahl and Bierbaum (1998), van Kraaij et al. (1999), Ennahar et al. (2000), Sablon et al. (2000), and McAuliffe et al. (2001).

BIOSYNTHETIC PATHWAY

All bacteriocins are synthesized as a biologically inactive prepeptide carrying an N-terminal leader peptide that is attached to the C-terminal propeptide. For lantibiotics, the serine, threonine, and cysteine residues in their propeptide parts undergo extensive posttranslational modification to form Lan/MeLan. The biosynthetic pathway of lantibiotics follows a general scheme (as shown in Figure 13.1): formation of prepeptide, modification reactions, proteolytic cleavage of the leader peptide, and the translocation of the modified prepeptide or mature propeptide across the cytoplasmic membrane. The cleavage of the leader peptide may take place prior to, during, or after export from the cell. Based on the biosynthetic pathway, two groups of genetic organization of lantibiotics can be identified (Sahl and Bierbaum, 1998; Guder et al., 2000; McAuliffe et al., 2001). In the producers of the group I lantibiotics, as in the case of nisin, epidermin, subtilin, and Pep5, the dehydration reaction is presumably catalyzed by the LanB enzyme, whereas LanC is involved in the thioether formation. The modified prepeptide is processed by a serine protease LanP and translocated through the ABC-transporter LanT. In contrast, lantibiotics of group II, as in the case of cytolysin, lacticin 481, and mersacidin, are very likely modified by a single LanM enzyme (van Kraaij, 1999; McAuliffe et al., 2001) and processing takes place concomitantly with transport by LanT(P). Lactocin S is the exception to this classification. It is modified by a single LanM enzyme and processing takes place prior to export and may therefore represent a new group (Skaugen et al., 1997).

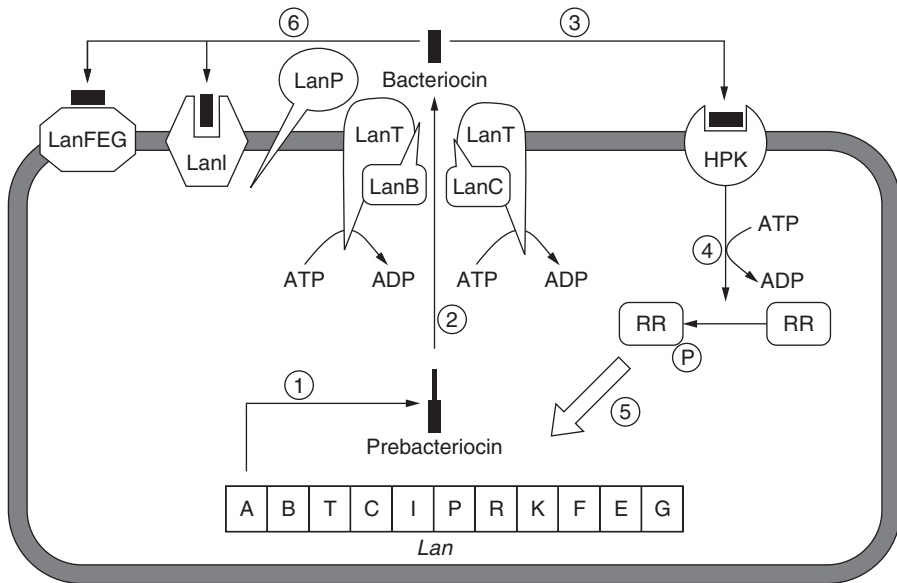


FIGURE 13.1 A schematic diagram of the biosynthesis of lantibiotics. 1: Formation of prebacteriocin. 2: The prebacteriocin is modified by LanB and LanC, translocated through a dedicated ABC-transporter LanT, and processed by LanP, resulting in the release of mature bacteriocin. 3: Histidine protein kinase (HPK) senses the presence of bacteriocin and autophosphorylates. 4: The phosphoryl group (P) is subsequently transferred to the response regulator (RR). 5: RR activates transcription of the regulated genes. 6: Producer immunity.

With the exception of class IIc bacteriocins, which are produced with a typical N-terminal signal sequence of the *sec*-type and processed and secreted through the general secretory pathway (Leer et al., 1995; Worobo et al., 1995), class II bacteriocins are synthesized as a prepeptide containing a conserved N-terminal leader and a characteristic double-glycine proteolytic processing site. Unlike the lantibiotics, they do not undergo extensive posttranslational modification. Following the formation of prepeptide, the prepeptide is processed to remove the leader peptide concomitant with export from the cell through a dedicated ABC-transporter and its accessory protein (Nes et al., 1996; Ennahar et al., 2000). The biosynthetic pathway of class II bacteriocins is shown in Figure 13.2.

Several functions of the leader peptides have been proposed. They may serve as a recognition site that directs the prepeptide toward the maturation and transport proteins, protects the producer strain by keeping the lantibiotic in an inactive state while it is inside the producer, and interacts with the propeptide domain to ensure a suitable conformation essential for enzyme–substrate interaction (van der Meer, 1994; van Belkum et al., 1997; Sablon et al., 2000; McAuliffe et al., 2001).

POSTTRANSLATIONAL MODIFICATION, ACTIVATION, AND TRANSPORT

A two-step posttranslational modification reaction of a prelantibiotic leading to formation of Lan/MeLan was first proposed by Ingram (1969; 1970). In the first step, the hydroxyl amino acids serine and threonine are dehydrated to yield 2,3-didehydroalanine or 2,3-didehydrobutyrine, respectively. Some dehydrated amino acids do not contain cysteine residues and remain as such in the mature peptide; others undergo an intramolecular Michael addition reaction that involves the thiol groups of neighboring cysteine residues and the double bonds of the didehydroamino acids, resulting in the formation of thioether bridges.

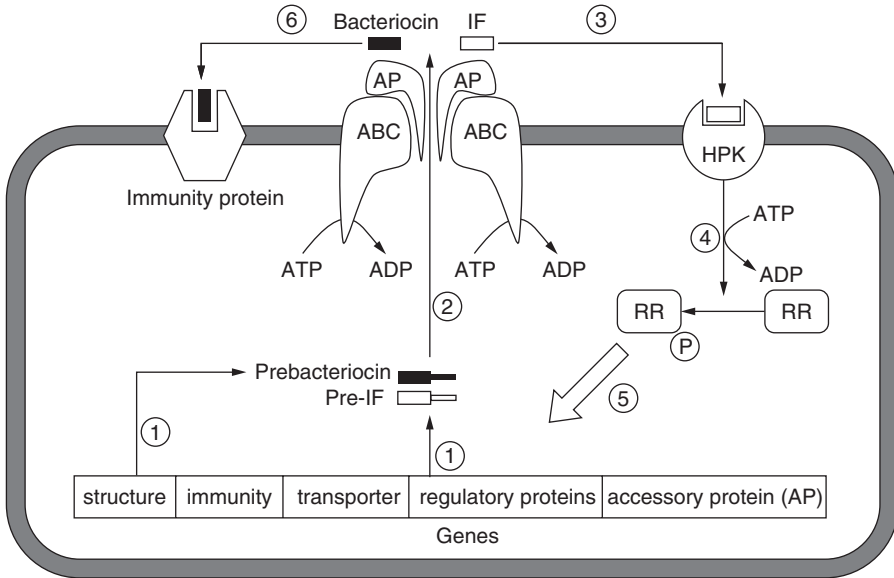


FIGURE 13.2 A schematic diagram of the biosynthesis of class II bacteriocins. 1: Formation of prebacteriocin and prepeptide of induction factor (IF). 2: The prebacteriocin and pre-IF are processed and translocated by the ABC-transporter, resulting in the release of mature bacteriocin and IF. 3: Histidine protein kinase (HPK) senses the presence of IF and autophosphorylates. 4: The phosphoryl group (P) is subsequently transferred to the response regulator (RR). 5: RR activates transcription of the regulated genes. 6: Producer immunity.

Following the modification reactions, the modified prelantibiotics undergo proteolytic processing to release the leader peptide that leads to activation of the lantibiotic. For group I lantibiotics, the removal of the leader peptide is performed by a serine protease LanP, and depending on the location of LanP this can take place before or after the peptide is exported from the producing cell via a dedicated ABC-transporter LanT. For example, the proteases LanP of epicidin 280 and Pep5 (Sahl and Bierbaum, 1998) are located intracellularly and proteolytic processing takes place within the cell. In contrast, the proteases of nisin (van der Meer et al., 1993) and epidermin (Geissler et al., 1996), which are located extracellularly, activate the lantibiotics only after export by the ABC-transporter. The ABC-transporter contains 500 to 600 amino acids and is characterized by two membrane-associated domains. The N-terminal domain consists of six membrane-spanning helices that can recognize the substrate and form its pathway across the membrane, whereas the cytoplasmic C-terminal domain contains two ATP-binding domains with the conserved ATP-binding or Walker motif. ATP hydrolysis, which likely occurs at the ATP-binding domains, provides the energy for the export process (Fath and Kolter, 1993; McAuliffe et al., 2001). The LanB and LanC enzymes, together with LanT transporter, probably form a multimeric membrane-associated complex (Siegers et al., 1996; Kiesau et al., 1997). For group II lantibiotics, which possess a conserved double-glycine cleavage site, proteolytic processing takes place concomitantly with export through a hybrid ABC-transporter. This unique ABC-transporter possesses an N-terminal protease domain of approximately 150 amino acid residues that cleaves the double-glycine leader (Nes et al., 1996; Sablon et al., 2000). This is exemplified in Figure 13.3.

The leader peptides of class IIa and b share considerable similarities with those of group II lantibiotics and contain the characteristic double-glycine cleavage site (Nes et al., 1996; Ennahar et al., 2000). The conservation of the cleavage site strongly suggests that the mechanism of processing and translocation of class IIa and b bacteriocins is very similar to that of the group II lantibiotics. Class IIc bacteriocins are processed by a signal peptidase during translocation across the cytoplasmic membrane.

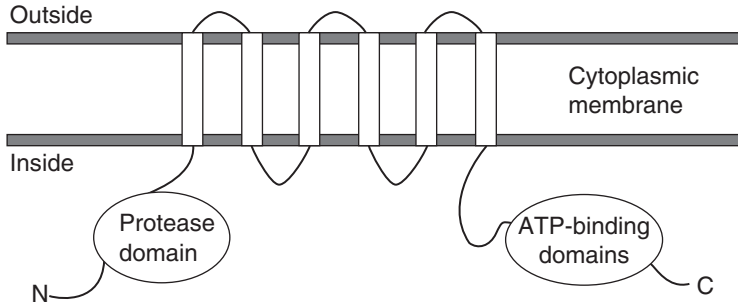


FIGURE 13.3 ABC-transporter with an N-terminal protease domain.

REGULATION OF BIOSYNTHESIS

Regulation of the biosynthesis of lantibiotic and nonlantibiotics is usually achieved through well-known two-component regulatory systems. These regulatory systems consist of two signal-producing proteins, a membrane-bound histidine protein kinase (HPK) and a cytoplasmic response regulator (RR) (Stock et al., 1989; Parkinson, 1993; Nes et al., 1996). In this signal transduction pathway, HPK autophosphorylates the conserved histidine residue in its intracellular domain when it senses a certain concentration of bacteriocin in the environment. The phosphoryl group is subsequently transferred to the conserved aspartic acid residue on the RR receiver domain and the resulting intramolecular change triggers the response regulator to activate the transcription of the regulated genes. These regulated genes include the structural gene, the export genes, the immunity genes, and in some cases the regulatory genes themselves (Kuipers et al., 1998).

The bacteriocin molecule itself may act as an external signal, as in the case of nisin and subtilin, autoregulating its own biosynthesis via signal transduction (Kuipers et al., 1995; Guder et al., 2000). In contrast, most strains producing class II bacteriocins may produce a bacteriocin-like peptide with no antimicrobial activity and use it as an induction factor (IF) to activate the transcription of the regulated genes. The IF is a small, heat-stable, cationic, and hydrophobic peptide that is first synthesized as prepeptide with a double-glycine leader sequence. A dedicated ABC-transporter specifically cleaves the leader peptide of IF concomitant with export of the mature peptide from the cell. The secreted IP acts as an external signal that triggers the transcriptions of the genes involved in bacteriocin production, as shown in Figure 13.2 (Nes et al., 1996; Ennahar et al., 2000).

PRODUCER IMMUNITY

The antimicrobial activity of a bacteriocin forces the producers to develop a protection system against their own bacteriocin (Abee, 1995). Two systems of lantibiotic immunity have been identified. Protection can be mediated by immunity proteins, LanI, and dedicated ABC-transport proteins, LanFEG, which can be encoded on multiple open reading frames (Reis et al., 1994; Siegers and Entian, 1995; Peschel and Gotz, 1996; Saris et al., 1996; McAuliffe et al., 2001). These two immunity systems work synergistically to protect the producing cells from their own bacteriocin (Klein and Entian, 1994). LanI, which is most likely attached to the outside of the cytoplasmic membrane, probably confers immunity to the producer cells by preventing pore formation by the bacteriocin of the producer. The LanFEG could act by transporting bacteriocin molecules that have inserted into the membrane back to the surrounding medium and thus keeping the concentration of the bacteriocin in the membrane under a critical level.

The immunity gene in class II bacteriocins usually codes a dedicated protein that is loosely interacted with the cytoplasmic membrane. The immunity protein, which is cationic and ranges in size from 51 to 254 amino acids, provides total immunity against the bacteriocin of the producer.

It is speculated that the interaction of immunity protein with the membrane protects the producer against its bacteriocin (Nissen-Meyer et al., 1993b; Venema et al., 1994; Nes and Holo, 2000).

MODE OF ACTION

Most bacteriocins of LAB interact with anionic lipids that predominate in the cytoplasmic membrane of Gram-positive bacteria leading to pore formation (Abee, 1995; Moll et al., 1999). Conductivity and stability of pores induced by class I lantibiotics may be heightened by docking molecules (lipid II, the peptidoglycan precursor), whereas in the case of class II bacteriocins receptors in the target membrane apparently act to determine specificity (Venema et al., 1995b,c). Class I bacteriocins may induce pore formation according to a wedge-like model, and class II bacteriocins may function by creating barrelstave-like pores or by creating a carpet mechanism whereby peptides orient parallel to the membrane surface and interfere with membrane structure (Moll et al., 1999).

Nisin is likened to a surface-active cationic detergent, with adsorption to the bacterial cell envelope the necessary first step of membrane disruption, followed by the inactivation of sulfhydryl groups. Bruno et al. (1992) showed nisin to completely dissipate the basal proton-motive force of *L. monocytogenes* Scott A. Other strains of *L. monocytogenes* were found to be equally sensitive to nisin and exhibit similar disruption to the pH gradient and membrane potential of the cells. Other class I bacteriocins of LAB behave in a similar manner. Carnocin UI49 acts upon the cytoplasmic membrane in a manner very similar to nisin (Stoffels et al., 1994). Ion permeability or channel formation in cytoplasmic membranes caused by bacteriocins of *L. acidophilus* was demonstrated by Palmeri et al. (1999) using artificial lipid membranes. Increases in membrane conductance were detected and channels with elementary conductance of 68 to 70 pS were measured by applying external voltages of different polarity.

In earlier research, Upreti and Hinsdill (1975) found lactocin 27, produced by *L. helveticus* LP27 and described as a bacteriocin whose effect was bacteriostatic, adsorbed equally well to sensitive and resistant cells terminating protein synthesis; however, there were substantial effects on DNA synthesis, RNA (ribonucleic acid) synthesis, or levels of ATP. With the lactostrepcin, Las 5, Zajdel et al. (1985) found the bacteriocin to immediately block syntheses of DNA, RNA, and protein, but these responses are probably a secondary reaction to severe membrane disruption and loss of intracellular constituents. For Las 5, both sensitive and insensitive cells adsorbed the bacteriocin equally well. Protoplasts of sensitive strains were not affected by Las 5, indicating that cell wall receptors were required for the action of the bacteriocin.

In 1986, Andersson demonstrated that resistant Gram-negative cells of *E. coli*, *Erwinia carotovora*, *P. aeruginosa*, and *S. marcescens* could be made sensitive to the bacteriocin of a strain of *L. plantarum* by transforming the Gram-negative cells to spheroplasts. It is now established that inactivation of Gram-negative pathogens with bacteriocins produced from Gram-positive bacteria normally requires the use of chelating agents (i.e., ethylenediamine tetraacetic acid [EDTA]) that function to diminish the barrier properties provided by the outer LPS membrane of Gram-negative bacteria (Stevens et al., 1991; Shefet et al., 1995; Scanell et al., 1997; Helander et al., 1997). Additional stress to Gram-negative bacteria as in a hurdle approach also can enhance the effectiveness of a bacteriocin. Ganzle et al. (1999a) demonstrated that curvacin A and nisin in combination with low pH, greater than 5% NaCl, or propylparaben also leads to an increased sensitivity of *Salmonella enterica* and *E. coli* toward these bacteriocins. So it would appear that combination packages of bacteriocins, processes, and other additives can significantly broaden the target spectrum of some LAB bacteriocins into the Gram-negative segment.

DETERMINATION OF BACTERIOCIN ACTIVITY

Bacteriocins are only one category of substances produced by bacteria that are inhibitory to other bacteria. Demonstration of antagonism from one strain of bacteria against another is very common. As early as 1676, Antonie van Leeuwenhoek documented antibiosis; the product from one microorganism inhibited growth of another (Joerger et al., 2000). Louis Pasteur and J.F. Joubert reported the inhibitive effect of common urine bacteria on *Bacillus anthracis* in 1877, but there are other compounds from bacteria and competitive situations whereby one type of bacterium can antagonize another. Besides bacteriocins, possible inhibitors produced by bacteria include clinical or therapeutic low-molecular-weight antibiotics (e.g., tetracyclines), lytic agents, toxins, bacteriolytic enzymes, bacteriophage, and other metabolic by-products, such as hydrogen peroxide and diacetyl. Also, in any ecologic niche, one type of bacteria can be more competitive than another regarding nutrient uptake or sensitivity to an environmental factor, such as oxygen. With such a wide range of possible inhibitory products or conditions, it is no wonder that some bacteriocin-like activities may not be caused by bacteriocins at all. When investigating bacteriocins synthesized by LAB, one must always be aware of the presence of relatively high amounts of organic acids and take appropriate steps to neutralize their activities.

There are many different laboratory methods to detect bacteriocin-like antagonism. Many issues surround the means of expressing inhibition. Questions that can be asked include the following: Should the test be direct or deferred? What is the effect of environmental pH, buffering capacity, nutrient content, redox potential, or protein-binding capacity? How were the producing culture and indicator strain handled? How long was the assay incubated? Is the bacteriocin inducible? How important is cell density or quorum sensing? Is the bacteriocin freely diffusing? Is the bacteriocin heat sensitive or highly prone to degradation by common proteolytic enzymes? How is bacteriocin activity measured (e.g., by units or zones or optical readings density)? Does the laboratory test relate to the intended commercial application? Obviously, many procedural factors affect the inhibitive activity of a strain. Mayr-Harting et al. (1972), Piddock (1990), and Parente and Riccardi (1999) have reviewed a number of methods for detection and measurement of bacteriocin activity.

AGAR PLATES

A common means for screening bacteriocin activity is the use of agar media contained in petri plates. There are many variations, most of them derivatives of the “spot-on-lawn” approach, which can involve an agar overlay. The direct simultaneous tests are the simplest. The producing and indicator cultures are incubated concurrently before examination for zones of inhibition around the producing strains. An example is the addition of indicator cells to tempered, molten agar used to prepare pour plates in which the producing strain is spot-inoculated onto the hardened agar (Sabine, 1963).

Deferred methods are often more sensitive than direct tests and allow separation of the independent variables of time and conditions of incubation for the producing and indicator strains (Tagg et al., 1976). The assay described by Kekessy and Piguet (1970) is an example of a procedure in which the producing and indicator strains can each be grown on different optimal media. The producing culture is spot inoculated onto a spread plate; after growth the agar mass is aseptically dislodged with a spatula from the petri dish bottom and transferred to the lid of the dish by striking the closed dish onto the bench top until the agar flips down onto the lid. A soft agar overlay seeded with the indicator is then poured over the inverted agar, and following reincubation, bacteriocin-positive cultures display a halo of clearing in the lawn around the original button of growth. Although requiring more time and manipulation and more susceptible to “smearing” caused by condensation within the petri dish, the Kekessy-Piguet assay is a sensitive plate test that minimizes the effects of acids and bacteriophage because the producing and indicator strains are physically separated by a layer of agar.

The composition of the agar medium is an important factor in any of these plate assays for bacteriocin activity. Lindgren and Clevstrom (1978) suggested that diffusion methods using agar or other gelling agents may not be suitable for assaying bacteriocin activity because the antibacterial agent may not be diffusible. For example, we found the bacteriocin activity of *Pediococcus acidilactici* PO2 to be severely diminished with use of M17 agar as the soft agar overlay using the Kekessy-Piguet method (Hoover et al., 1989). Apparently, a buffering compound in the medium, α -glycerol phosphate, bound the protein; however, Spelhaug and Harlander (1989), using three different plating assays, found no difficulties demonstrating bacteriocin activity with *Pediococcus pentosaceus* FBB61 and FBB63-DG2 in M17-glucose agar. It is probable that different bacteriocins were produced by these strains of *P. acidilactici* and *P. pentosaceus*.

To demonstrate the production of bacteriocins in or on meat products, Saucier and Greer (2001) adapted an agar diffusion assay using *Carnobacterium piscicola* LV17 as the bacteriocin producer and *Carnobacterium divergens* LV13 as the indicator strain. Two meat models were used, a commercial cooked meat medium and lean and fat disks (10 cm²) of pork. *C. piscicola* LV17 could be grown at relatively low levels (<10⁴ CFU/cm² or g) on the meat portions (except for the disks of lean meat) and overlaid with a soft agar containing the indicator to detect bacteriocin production. Strain LV17 could grow on the lean pork sections, but bacteriocin production was not demonstrable.

NEWER METHODS

Mass spectrometry was adapted for the rapid detection of bacteriocins by Rose et al. (1999) using pediocin, nisin, brochocin A and B, and enterocin A and B from culture supernatants. The method, called matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), was originally devised for the examination of large molecules, especially biopolymers. A 30-second water wash of the supernatant was effective in removing interfering compounds; however, the procedure requires further development to accurately identify bacteriocins in foods that by their nature contain a myriad of contaminating and interfering substances.

Polymerase chain reaction (PCR) methods have been used to detect genes responsible for bacteriocin production and regulation in bacterial cultures. For example, Rodriguez et al. (1995) amplified a 75-bp gene fragment of the lactocin S structural gene in seven bacteriocinogenic strains of lactobacilli isolated from fermented sausages. In an isolate of *L. lactis* subspecies *lactis* from raw milk cheese, Garde et al. (2001) detected the genes necessary for synthesis of lacticin 481 and nisin using PCR techniques with specific probes.

Dot-blot and colony hybridization was used by Rodriguez et al. (1998) to detect enterocin AS-48 from enterococci isolated from milk and dairy products. Genes encoding for synthesis of AS-48 have already been sequenced (Martinez-Bueno et al., 1994) and PCR developed for rapid detection of these genes in isolated strains (Joosten et al., 1997). Colony hybridization allowed for a more rapid technique in which cultures are spotted on MRS (de Man de Man-Rogosa-Sharpe) agar, cultures are incubated, and colony growth is transferred to Hybond N+ membranes for lysis with subsequent hybridization using a DNA probe.

Mugochi et al. (2001) developed a rapid and sensitive assay method for detection of bacteriocins in fermentation broth. In a flow cell, low concentrations of potassium ion could be measured, so that released potassium ions from a bacteriocin-sensitive indicator strain directly correlate to concentrations of crude bacteriocin present in fermentation broth injected into the cell. This method compared very well to the conventional agar well diffusion assay.

PRODUCTION AND MODELING

To produce bacteriocins on a commercial basis, optimization of yield is desirable. In a study of nisin, pediocin AcH from *P. acidilactici*, leuconocin Lcm1 by *Leuconostoc carnosum*, and sakacin A by *L. sake* Lb 706, Yang and Ray (1994) found that in general growth medium the key factors

were maintaining optimum pH and supplementing specific nutrients for each strain or species. Essentially, the conditions favored high cell density and resulted in a comparable bacteriocin yield, indicative of a primary metabolite. To further enhance yield and purity of LAB bacteriocins from industrial fermentations and lower production costs, Coventry et al. (1996) demonstrated that nisin, pediocin PO2, brevicin 286, and piscicolin 126 could be significantly free of contaminating proteins (92% to 99%) as compared with original culture supernatants and ammonium sulfate preparations by using food-grade diatomite calcium silicate and several desorbing agents.

An important part of the successful development for bacteriocin use rests on the infusion of biotechnologic techniques, such as strain improvement and protein engineering, as well as optimization of production methods to reduce costs. Following this path, Carolissen-Mackay et al. (1997) reviewed protocols for purification of bacteriocins from LAB. To reduce production costs, improvements in speed and yield of purification methods for bacteriocins from culture broth continues (Guyonnet et al., 2000).

To optimize the application of bacteriocins, models have been devised to predict the commercial effectiveness of bacteriocins based on information gathered in test systems. Using traditional agar plates, Blom et al. (1997) designed a method for simultaneous examination of the effect of intrinsic factors (pH, concentration of indicator cells, agar, soy oil, and sodium chloride concentration) on the diffusion and efficacy of bacteriocins. They tested sakacins A and P, piscicolin 61, pediocin PA-1, and nisin, and found that each of the bacteriocins had a unique intrinsic factor effect profile, although pH and the concentration of indicator cells similarly affected all of the bacteriocins. Except for use of a commercial preparation of nisin, the other bacteriocins were produced using *L. sake* Lb790, modified to produce each of the remaining bacteriocins. Parente et al. (1998) used logistical regression analysis of categorical data to generate predictive models for the probability of survival for *L. monocytogenes* versus leucocin F10 and nisin in TSB with 0.6% yeast extract. Nisin had a significant effect on the probability of survival, but supplementation with leucocin F10 was necessary to eliminate *L. monocytogenes*. Lower pH values in the broth decreased survival, whereas NaCl and EDTA demonstrated a limited effect. In a somewhat different approach, Pleasants et al. (2001) modeled the growth of *L. monocytogenes* L70 in response to *L. sake* 706, a producer of sakacin A. In MRS broth it was found that the *L. sake* strain grew much faster than *L. monocytogenes* L70 at reduced pH. Excretion of sakacin A by its producer rapidly reduced the presence of *L. monocytogenes* in mixed culture. It was deemed that the model could accurately predict growth and interaction of the two bacteria in the test medium.

RESISTANCE

As has been well established in microbial ecology, the presence of an antimicrobial substance will eventually select for resistant varieties of bacteria in a given environment. As with therapeutic antibiotics, bacteriocin-resistant mutants have arisen. Gravesen et al. (2002) examined the responses of a number of strains of *L. monocytogenes* to pediocin PA-1 and nisin and found a wide range of resistances to the two bacteriocins occurring naturally. The influence of environmental stress (reduced pH, low temperature, and the presence of sodium chloride) was apparently bacteriocin-specific — that is, these stresses did not influence the frequency of resistance to pediocin PA-1, but the frequency of nisin resistance was significantly reduced. Also in contrast, the stability of the phenotype of nisin resistance varied substantially, whereas resistance to the pediocin was stable with ongoing growth of *L. monocytogenes*. Fitness costs as measured by reduced growth rate in pediocin-resistant mutants were demonstrable, but nisin-resistant mutants showed limited growth rate reductions. Lastly, it was found that the bacteriocin-resistant mutants of *L. monocytogenes* were not more sensitive to the applied environmental stresses than wild-type strains; in a model sausage system growth differences were minimal.

Horn et al. (1998, 1999) moved the genes of pediocin PA-1 into nisin-producing *L. lactis* FI5876. As a result both pediocin PA-1 and nisin A were synthesized concurrently in the strain.

Production levels of pediocin PA-1 in *L. lactis* FI5876 were at the same levels as found in the parent, *P. acidilactici* 347. Because pediocin PA-1 and nisin A are unrelated bacteriocins, use of *L. lactis* FI5876 as a starter culture in fermented dairy products should prevent the emergence of any bacteriocin-resistant isolates of *L. monocytogenes* because the frequency for emergence of a strain resistant to both peptides should be low.

EXAMPLES OF ACTIVITY SPECTRA AND BIOCHEMICAL PROPERTIES

LACTOCOCCUS

The inhibition of commercial cheese starter cultures by similar dairy bacteria has been known since the early 1900s (Rogers and Whittier, 1928; Whitehead and Riddet, 1933). It was demonstrated that these effects were caused by inhibitory proteins (Mattick and Hirsch, 1947; Whitehead, 1933). At that time, the two best characterized proteins causing this phenomenon in lactococci were nisin and diplococcin. Nisin is the recognized standard for bacteriocins that other potential applicants for commercialization hope to follow. However, diplococcin (produced by some strains of *Lactococcus lactis* subspecies *cremoris*) lacks the effectiveness of nisin because of extreme instability when purified; an activity spectrum limited to other cultures of *L. lactis* (diplococcin is ineffective against sporeformers); and a relatively high degree of sensitivity to trypsin, pronase, and α -chymotrypsin (Oxford, 1944; Davey, 1981; Davey and Pearce, 1982). Diplococcin has a higher molecular weight than nisin, 5300 versus 3500 daltons (Davey and Richardson, 1981; Gross and Morell, 1967), and lacks the unusual sulfur-containing amino acids (lanthionine and α -methylanthionine) that nisin possesses (Davey and Pearce, 1980; Gross et al., 1969). Diplococcin also does not contain dehydroalanine or dehydrobutyrine (Davey and Pearce, 1982). In many ways, nisin has greater similarities to subtilin, a bacteriocin produced by *Bacillus subtilis*, than to diplococcin.

Apparently the majority of *L. lactis* subspecies *cremoris* strains do not synthesize diplococcin, as indicated in a study by Davey and Richardson (1981) in which only one of 150 strains was positive for the bacteriocin. Nonetheless, the capability of a culture to produce diplococcin is a significant concern to cheesemakers because diplococcin-positive strains rapidly predominate over other lactococci used in multiple-strain starter cultures (Davey and Pearce, 1980; Klaenhammer, 1988). Diplococcin-negative variants are desirable in such a situation; Davey and Pearce (1980) developed diplococcin-negative strains of *Streptococcus cremoris* 346 (now *L. lactis* subspecies *cremoris* 346) whose fermentative performance for cheesemaking was unaffected by loss of this unwanted characteristic. As with most other bacteriocins, diplococcin is normally associated with plasmid DNA. Davey (1984) found diplococcin biosynthesis mediated by a 54-megadalton (MD) conjugative plasmid in *S. cremoris* 346.

Lactostrepcins are bacteriocins produced by nonnisin-producing *L. lactis* subspecies *lactis* strains, almost all *Lactococcus lactis* subspecies *diacetylactis* strains, and some *L. lactis* subspecies *cremoris* strains (Kozak et al., 1978). The lactostrepcins function optimally only at acid pH (<5.0) and have a molecular weight of 10,000 or more. Although heat-stable (121°C for 10 minutes), they are especially susceptible to most proteolytic enzymes, as well as phospholipases A and C and lipases (Bardowski et al., 1979; Dobrzanski et al., 1982). According to the system of Bradley (1967), they were first categorized as group I bacteriocins.

Five types of lactostrepcins have been differentiated, primarily based on the range of bacteriocidal activity (Dobrzanski et al., 1982). Lactostrepcins are stabilized by enriched, complex growth media, such as milk, and have shown antagonism not only against lactococci but streptococci of groups A, C, and G; *Bacillus cereus*; and a few varieties of *Leuconostoc* and *Lactobacillus* (Zajdel and Dobrzanski, 1983).

Probably the most studied of the lactostrepcins has been lactostrepcin 5 (Las 5) as produced by *S. cremoris* 202. Like diplococcin, Las 5 contains no unusual amino acids, nor a lipid component

like other lactostrepcins. Its molecular weight has been estimated as 20,000 by Laemli and Favre (1973) and 6000 by Davey and Richardson (1981). Dobrzanski et al. (1982) searched for association of Las 5 synthesis with one of the plasmids of *S. cremoris* 202 but were unable to establish linkage. A similar attempt was made to link plasmids of *Streptococcus lactis* 71 with its bacteriocins, lactostrepcins 3 and 4, but the results were also inconclusive.

Bacteriocin typing with lactostrepcins has been investigated (Bardowski and Kozak, 1981). Lactostrepcin typing of lactococci appears possible because many of the strains are sensitive to at least one lactostrepcin. Seven different lactostrepcin types of lactococci can be determined, although lactostrepcin sensitivity does not correspond to taxonomic classification of these bacteria. Of potential benefit would be the correlation of industrially important lactococci with lactostrepcin type (Dobrzanski et al., 1982).

Lactococci are capable of producing other bacteriocins besides nisin, diplococcin, and the lactostrepcins. Geis et al. (1983) conducted a survey of bacteriocin activity encompassing 280 commercial isolates of lactococci. In comparing antagonism between broth extracts and direct agar plating, they found 14 positives in broth and 5 positives on agar for 54 strains of *S. lactis*, 36 positives in broth and 1 positive on agar for 93 cultures of *Streptococcus lactis* subspecies *diacetylactis*, and 15 positives in broth and 10 positives on agar for 133 strains of *S. cremoris*. Clearly there was a difference in expression of inhibiting activity between liquid and solid systems. Geis et al. (1983) defined 8 bacteriocin types based on sensitivity and immunity to other bacteriocin-positive cultures. Although some of the antagonistic reactions very closely resembled nisin or diplococcin, others did not; none were characteristic of the lactostrepcins. One group of nonnisin-producing strains was inhibitive versus species of *Clostridium*.

Lacticin 3147, a class II bacteriocin, is produced by a lactococcal strain originally isolated from kefir; it is a broad-spectrum bacteriocin consisting of two individual peptides genetically residing on a self-transmissible plasmid that can be easily moved to other strains of lactococci used as cheese starter cultures (Ross et al., 2000). In cottage cheese, a 3- \log_{10} reduction in CFU/g of *L. monocytogenes* was consistently shown using a lacticin 3147-producing culture of *L. lactis* subspecies *lactis*, whereas a control nonlacticin 3147-producing strain demonstrated no effect on *L. monocytogenes* (McAuliffe et al., 1999). On the surface of mold-ripened cheese a lacticin 3147-producing *L. lactis* subspecies *lactis* again reduced *L. monocytogenes* by 3 \log_{10} CFU/g, but this time as compared to a nisin-producing *Lactococcus* (Ross et al., 2000). Lacticin 3147 acts by selectively dissipating the membrane potential (McAuliffe et al., 1998).

Another antibacterial peptide produced by lactococci is bacteriocin S50. It has a relatively narrow spectrum of activity. Studied by Kojic et al. (1991), this bacteriocin is produced by *L. lactis* subspecies *diacetylactis* S50. It is active only against other *Lactococcus* species and is heat-stable (100°C for 60 minutes) and active in the pH range 2 to 11.

Not all investigations of antimicrobial action by dairy streptococci have involved the determination of specific bacteriocin effect. Pulusani et al. (1979) examined the activity of skim milks fermented by *Streptococcus thermophilus* as well as *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. There was strong inhibition of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *B. subtilis*, but bacteriocin activity could not be inferred. Reddy and Ranganathan (1983) measured the presence of antimicrobial substances in the cell-free filtrates from 14 strains of *S. lactis* subspecies *diacetylactis*. Subculturing resulted in mutant derivatives with inhibitory action that exceeded the parent activity. Greater inhibition was shown against Gram-negative bacteria than Gram-positive bacteria, suggesting the action of organic acids. With the exception of *Geotrichum candidum*, molds were not affected.

In *Lactococcus*, bacteriocin production has been used as a marker in plasmid transfer studies and in other genetic procedures. Neve et al. (1984) characterized bacteriocin plasmids from strains of *S. lactis*, *S. lactis* subspecies *diacetylactis*, and *S. cremoris*. Conjugal transfers were done into a plasmid-free recipient strain of *S. lactis* subspecies *diacetylactis*. The activity spectra of these

bacteriocins included strains of lactococci, lactobacilli, pediococci, *Streptococcus faecalis*, *Streptococcus sanguis*, *Leuconostoc* species, *B. subtilis*, and clostridia.

A bacteriocin produced by *S. lactis* subspecies *diacetylactis* WM₄ has been studied by Scherwitz et al. (1983). Bacteriocin production was conjugally transferred into *S. lactis* and found associated with an 88-MD plasmid. The bacteriocin from *S. lactis* subspecies *diacetylactis* WM₄ was active against a variety of lactococci. Cloning of restriction enzyme fragments of the 88-MD plasmid into a recombinant plasmid expressed in *S. lactis* resulted in greater inhibition versus the indicator strain of *S. diacetylactis*; this is an excellent example of enhanced production of a bacteriocin in the development of commercial dairy strains (Scherwitz-Harmon and McKay, 1987). Further work with WM₄ (Stoddard et al., 1992) involved the characterization of the lactococcin A gene cluster by DNA restriction fragment deletion, subcloning, and nucleotide sequence analysis. This molecular analysis suggests the existence of a signal sequence-independent secretory pathway in the lactococci that is similar to the secretion and processing system in *B. subtilis*.

van Belkum et al. (1992) cloned two bacteriocin genes from an *L. lactis* subspecies *cremoris* plasmid that specified both bacteriocin production and immunity. This culture, 9B4, was originally described by Geis et al. (1983). Two regions on the plasmid were identified that had different specified inhibitory activities toward *L. lactis* indicator strains; however, hybridization experiments revealed a high level of homology between the two bacteriocin gene regions. The authors noted the desirability for the isolation of bacteriocin production and immunity genes for use as food-grade vectors that are selectively retained within the plasmid-bearing population.

Further study of the two bacteriocin operons of the plasmid from *L. lactis* subspecies *cremoris* 9B4 by van Belkum et al. (1991) resulted in the sequencing and analysis by deletion and frameshift mutation of the regions responsible for bacteriocin production and immunity. It was found that the nucleotide sequences of both fragments upstream of the first detectable open reading frames and the first 20 base pairs of the first open reading frame of both bacteriocin operons appeared identical. From this finding, movement of the operons by transposition-like events was suggested because each fragment encoding for bacteriocin synthesis was flanked by similar nucleotide sequences.

As studies continue, the genus *Lactococcus* continues to supply bacteriocins active against *L. monocytogenes* and other Gram-positive pathogens. Isolated from raw pork, lacticin FS92 is another example; it contains 32 amino acids, is heat-stable, and as a food preservative appears desirable because resistance to it from strains of *L. monocytogenes* is low (Mao et al., 2001). Unlike pediocin PA-1, curvaticin FS47, and lacticin FS56, resistant mutants of *L. monocytogenes* remained sensitive to lacticin FS92. However, strain-to-strain variations are great whether considering resistance of target strains or effectiveness of bacteriocins. For example, Castellano et al. (2001) found susceptibility of strains of *L. monocytogenes*, *L. innocua*, and *L. seeligeri* to be strain-dependent at each pH that was examined in response to lactocin 705, enterocin CRL35, and nisin.

In a study using MRS agar to isolate lactic bacteria isolated from raw milks (ewe, goat, and cow) and survey bacteriocin production, the majority of the bacteriocin producers were lactococci (Rodriguez et al., 2000). Nisin-producing LAB were the most frequently encountered. Almost as abundant as the nisin producers were lactococci synthesizing lacticin 481 and enterococci producing AS-48. Using PCR techniques, nisin was found in 39 of the 67 bacteriocin-producing lactococci, with lacticin 481 found from 23 isolates. Antilisterial activity was well represented as was activity against a typical range of Gram-positive bacteria normally used in bacteriocin studies. This work demonstrates the relatively high incidence of bacteriocin-producing bacteria occurring in milk, primarily lactococci.

PEDIOCOCCUS

The pediococci share many characteristics with the lactococci, including the ability to produce a range of interesting bacteriocins. It was recognized in cucumber fermentations using *P. pentosaceus* and *Lactobacillus plantarum* as starter cultures that the pediococci inhibited growth of lactobacilli

(Etchells et al., 1964). Because *L. plantarum* is as resistant to acids as *P. pentosaceus* is, if not more so, it was assumed that other antagonistic substances besides organic acids were active. Further investigation by Fleming et al. (1975) resulted in the isolation and characterization of *P. pentosaceus* FBB61 and L7230. These cultures showed similar inhibitory activity against a number of Gram-positive bacteria (most notably the pathogens *Staphylococcus aureus* and *B. cereus*) but were not active versus Gram-negative bacteria, yeasts, or each other. Catalase was included in their assays to obviate hydrogen peroxide effects. The bacteriocin of *P. pentosaceus* FBB61 was characterized by Rueckert in 1979 (M.S. thesis, Michigan State University, East Lansing). It was bacteriocidal, heat-stable (100°C for 60 minutes), sensitive to pronase, and nondialyzable. Daeschel and Klaenhammer (1985) further examined the antagonistic activity and host cell immunity of strains FBB61 and L7230. The bacteriocin produced by these cultures, designated pediocin A, was effective against other pediococci, *Lactobacillus brevis* and *L. plantarum*; the nisin-producing ATCC 11454 *S. lactis*; four strains of *S. aureus*; and cultures of clostridia representing *C. botulinum*, *Clostridium perfringens*, and *Clostridium sporogenes*. The bacteriocin was associated with a plasmid of approximately 13.6 MD; however, as noted by Klaenhammer (1988), further study of this bacteriocin was hindered by the low levels produced and difficulties in purification, which curbed detailed biochemical characterization.

Graham and McKay (1985) found the apparent biosynthesis of a bacteriocin in *Pediococcus cerevisiae* FBB-63 linked to a plasmid of approximately 10.5 MD. Gonzalez and Kunka (1987) correlated production of bacteriocin activity, designated pediocin PA-1, with a 6.2-MD plasmid in *P. acidilactici* PAC 1.0. Antagonism was demonstrated against other pediococci, some lactobacilli, and a strain of *Leuconostoc mesenteroides* subspecies *dextranicum*. No activity was found against lactococci. Partially purified pediocin PA-1 was ineffective versus micrococci, staphylococci, and some lactobacilli. Pediocin PA-1 was proteinaceous and bactericidal in action and sensitive to protease, papain, pepsin, and α -chymotrypsin. It was heat-stable (100°C for 10 minutes) and, as determined by gel filtration, had a molecular weight of approximately 16,500.

Marugg et al. (1992) reported the cloning, expression, and nucleotide sequence of the genes involved in the synthesis of pediocin PA-1. The bacteriocin was associated with a 9.4-kbp plasmid. The genes were cloned and expressed in *E. coli*. Deletion analysis demonstrated that a 5.6-kbp fragment from the plasmid was necessary for production of the bacteriocin. Four clustered open reading frames were found in the 5.6-kbp fragment. The first encoded for the 62 amino acid precursor of pediocin PA-1, with amino acid residues 19–62 corresponding to the purified bacteriocin. Although functions could not be determined for the next two downstream open reading frames, the last gene specified a large protein of 724 amino acids required for pediocin PA-1 production in *E. coli* that probably corresponded to an ATP-dependent transport protein. Comparison of the amino acid sequence of pediocin PA-1 with leucocin A-UAL 187 (Hastings et al., 1991), lactacin F (Muriana and Klaenhammer, 1991a), lactococcin M (van Belkum et al., 1991), lactococcin A (Holo et al., 1991; van Belkum et al., 1991), and lactococcin B (van Belkum et al., 1992) showed few similarities, except for the N-terminal leaders of 18–24 amino acids of the precursor proteins, particularly near the cleavage site. This site appears to be uniquely conserved in bacteriocin precursors from LAB, suggesting the requirement for separate processing enzyme(s) in the maturation of small heat-stable bacteriocins synthesized by LAB.

Hoover et al. (1988) examined bacteriocin activity associated with a plasmid of approximately 5.5 MD detectable in *P. acidilactici* P02, B5627, and PC and *P. pentosaceus* MC-03. Strain MC-03, isolated from pepperoni, was speciated primarily on the basis of growth temperature characteristics. Besides antagonism against other pediococci, *S. aureus*, *B. cereus*, *S. faecalis*, and *L. mesenteroides*, four of five strains of *L. monocytogenes* were also inhibited, as shown by agar plate diffusion assays. *P. acidilactici* PAC 1.0 shares a common heritage with *P. acidilactici* B5627 (Carlos Gonzalez, personal communication), and as found by rDNA typing, strains P02, PC, and B5627 are essentially the same genetically (Susan Harlander, personal communication). Most of the commercial starter cultures of *Pediococcus* used to manufacture fermented meat products in

the United States have the capability of producing a bacteriocin with the potential to inhibit several important Gram-positive foodborne pathogens.

Hoover et al. (1989) surveyed 37 cultures of pediococci for antagonistic effects against eight strains of *L. monocytogenes* using an agar plate diffusion overlay method. There was an observable or positive response in 28% of the matchings, indicating that a bacteriocin effect of these LABs versus *L. monocytogenes* may not be limited to a few industrial starter cultures. Indeed, five strains of pediococci that were isolated from the gastrointestinal tract of humans inhibited all the *L. monocytogenes* cultures. These strains of pediococci were not only of interest because of their overall effectiveness against the *L. monocytogenes* cultures but also because of their site of isolation. Members of the genus *Pediococcus* are not considered pathogenic; however, these strains were isolated from surgical treatment of human abdominal disease (William Riebel, personal communication). Although these pediococci were probably opportunistic in their presence and may not have contributed to the disease syndrome, their presence in such an environment was nonetheless unexpected.

Hoover et al. (1989) also more closely examined the inhibition of *L. monocytogenes* 19113 by dialyzed growth supernatant of *P. acidilactici* P02. Besides ruling out the inhibitive effects of lactic acid, the emergence of *L. monocytogenes* growth over time was also evident using a liquid assay. Apparently, prolonged exposure of the sensitive bacteria to the bacteriocin either resulted in the selection of a resistant subpopulation of *Listeria* or the bacteriocin was bound or made unavailable so that the bacteriocin-sensitive population could then reestablish itself.

Pucci et al. (1988) prepared a dried powder of growth extract from *P. acidilactici* PAC 1.0 amended with 10% nonfat dry milk. Against *L. monocytogenes* LM01 they found the PA-1 powder effective over a pH range of 5.5 to 7.0 at both 4°C and 32°C. PA-1 powder acted as an effective preservative against an inoculum of approximately 10-10⁷ CFU/ml of *L. monocytogenes* in cottage cheese, half-and-half cream, and cheddar cheese soup for 2 weeks at 40°C. Lysis of the *L. monocytogenes* was evident.

Pediocin AcH was characterized by Bhunia et al. (1987; 1988), and optimal conditions for its production were established (Biswas et al., 1991). Synthesized by *P. acidilactici* H, pediocin AcH was approximately 2700 daltons, sensitive to proteolytic enzymes (trypsin, papain, ficin, and chymotrypsin and proteases IV, XIV, XXIV, and K), heat-resistant (121°C/15 minutes), resistant to a range of organic solvents, and effective over a pH range of 2.5 to 9.0. A rather wide range of activity was noted. Although only single strains of some genera were examined by Bhunia et al. (1988), inhibition by pediocin AcH of Gram-negative bacteria was reported (*Aeromonas hydrophila* and *Pseudomonas putida*). Inhibition of *A. hydrophila* was also found with culture derivatives of *P. pentosaceus* FBB61/L7230 (Thomas Montville, personal communication). This is of interest because bacteriocins produced by Gram-positive bacteriocin commonly have no effect against Gram-negative bacteria.

A 7.4-MD plasmid harboring the genes for activity and immunity of pediocin AcH from *P. acidilactici* H was conjugated into another strain of *P. acidilactici* by Ray et al. (1989a). For *P. acidilactici* strains E, F, and H, bacteriocin activity was associated with plasmids of 7.4 to 8.0 MD (Ray et al., 1989b). Overall, the strains contained a total of two to three plasmids ranging from 7.4 to 40.2 MD in size. Association of carbohydrate fermentation and antibiotic resistance could not be made with any of the detectable plasmids. The authors noted that mutanolysin was more effective than the more often used lysozyme for lysing cells of pediococci.

Given the use of pediococci as a meat starter culture by the food industry, the effectiveness of bacteriocin-producing pediococci against *L. monocytogenes* has been investigated in meat systems. Berry et al. (1990) evaluated *Pediococcus* species strains JD1-23 and MP1-08 versus *L. monocytogenes* Scott A in summer sausage chubs using two different spice blends. Inoculated at an initial level of about 10⁶ CFU/g, *L. monocytogenes* Scott A was reduced approximately 2 log₁₀ cycles using a bacteriocin-positive culture and approximately 1 log₁₀ cycle using a bacteriocin-negative isolate; however, recovery of Scott A using McBride *Listeria* agar was intermittent and did not

indicate that the bacteriocin was effective for inactivation of Scott A that survived the smoking process given the sausages. In a subsequent study (Berry et al., 1991), bacteriocin-producing *P. acidilactici* was found effective against *L. monocytogenes* in frankfurters.

Nielsen et al. (1990) examined strain Scott A in a fresh meat system. The bacteriocin producer was *P. acidilactici* from a commercial culture identified as Lactacel 110. Radiation-sterilized fresh lean beef muscle cut into small pieces represented the model food system. Their approach was to evaluate the bacteriocin present in a filter-sterilized growth extract against *L. monocytogenes* on the meat surface. Cut meat pieces were exposed to various buffer solutions containing bacteriocin and growth extract and strain Scott A in alternating sequences. Using this protocol Nielsen and coworkers demonstrated a reduction of attached *L. monocytogenes* by 0.5 to 2.2 log₁₀ cycles within 2 minutes of treatment with the bacteriocin. Meat pieces treated initially with the bacteriocin showed 1.0 to 2.5 log₁₀ cycle decreases in attached bacteria. The bacteriocin was functional on meat surfaces after a month of refrigerated storage.

Harris et al. (1989) examined 15 strains representing the genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, and *Leuconostoc* for inhibition against eight strains of *L. monocytogenes*. From these 15 lactic cultures, only cell-free supernatants from *Lactobacillus* species UAL11, *P. acidilactici* PAC 1.0, and *Leuconostoc* species UAL14 inhibited all eight strains of *L. monocytogenes* as measured by deferred antagonism and well diffusion methods. This inhibition was prevented by the addition of proteolytic enzymes. All cultures of *L. monocytogenes* that were examined showed an identical response to the bacteriocins.

Foegeding et al. (1992) showed that pediocin production by meat starter cultures enhanced safety against growth of *L. monocytogenes* during both the fermentation and drying portions of dry fermented sausage manufacture. Use of a bacteriocin-producing strain of *P. acidilactici* P02 effectively inactivated a mixture of five strains of *L. monocytogenes* (inoculated to a level of approximately 10⁵ CFU/g) when the pH at the end of the fermentation segment of the process was less than 4.9.

Further effort to optimize pediocin AcH as a food preservative was attended by Motlagh et al. (1992b). Sterile ground beef, sausage mix, cottage cheese, ice cream, and reconstituted dry milk food systems were investigated using *Listeria ivanovii* and *L. monocytogenes* as target organisms. They found that the greatest bactericidal effect of pediocin AcH occurred within 1 hour and was not significantly affected by food type, the *Listeria* strains that were examined varied in sensitivity to the bacteriocin, and the surviving cells of *Listeria* were capable of replicating in the presence of residual AcH during storage at 4°C and 10°C.

In an effort to improve the effectiveness of pediocin AcH against *L. monocytogenes*, Degnan and Luchansky (1992) used liposome encapsulation of the bacteriocin. In heated slurries of beef tallow and beef muscle, encapsulation of pediocin AcH within phosphatidylcholine liposomes improved bacteriocin activity nearly 30% compared with slurries containing free pediocin.

The pediocin family of bacteriocins are not contained in *Pediococcus* or even the LAB (pediocins have been found in *Leuconostoc*, *Lactobacillus* and *Carnobacterium*; Atrih et al., 2001). Coagulin, a peptide of 44 amino acids produced by *Bacillus cereus*, differs from pediocins AcH and PA-1 by only one amino acid in the C terminus (LeMarrec et al., 2000). The 3.5-kb operon of four genes for coagulin is plasmid-encoded. An isolate of *Lactobacillus plantarum* WHE 92 isolated from Muenster cheese was found to produce a bacteriocin identical to pediocin AcH produced from *P. acidilactici* H (Ennahar et al., 1996). WHE 92 was identified at the DNA level. It was found that pediocin AcH was produced more effectively in *L. plantarum* in the pH range of 5.0 to 6.0 than in *P. acidilactici* H. The authors noted that because dairy products are normally higher than pH 5.0, *L. plantarum* WHE 92 offers a more effective means of antagonism against *L. monocytogenes*. WHE 92 grew very well in cheeses. Clearly, the same bacteriocin in LAB can be expressed across a range of different genera.

Pediocin binding to target membranes has been shown to be related to electrostatic interactions by Chen et al. (1997a,b). Pediocin PA-1 can function in the absence of a protein receptor. Positive

patches of amino acid residues of pediocin PA-1 bound to negatively charged phospholipid head groups in the target membrane. Low pH strengthened interaction with the membrane by increasing the net charge of the bacteriocin; low ionic strength (i.e., low salt) enhances pediocin activity as high salt concentrations weaken the electrostatic interactions. Pediocin N5p, produced by a wine isolate of *P. pentosaceus*, nonspecifically absorbed to both Gram-positive and Gram-negative bacteria whether or not the cells were sensitive or resistant to it; however, for sensitive cells, higher values up to 30% imply the presence of lethal receptors (Manca de Nadra et al., 1998). Cell death occurred without lysis and the cations of magnesium and manganese were found to improve pediocin binding by 80% to 100%. In the case of pediocin N5p, a lipid moiety plays a key role in antagonism as suggested by the inactivation of N5p by lipase and chloroform.

PCR random mutagenesis was used to create amino acid substitution mutants of pediocin AcH (Miller et al., 1998). Seventeen mutations of AcH were isolated having 14 of the 44 amino acids substituted. Most of the mutations were inactive or demonstrated reduced antagonism. One mutant peptide showed a 2.8-fold higher activity against the indicator strain (*L. plantarum* NCD0955). The critical amino acids necessary for activity of the pediocins were a few basic amino acids and nonpolar amino acids in the hydrophobic C-terminal region. Structural models were proposed. For pediocin PA-1, there is a loss of activity on storage at refrigeration or ambient temperatures. Johnsen et al. (2000) engineered increased stability for the peptide by replacing a methionine residue (Met 31) with alanine, isoleucine, or leucine for protection from oxidation.

LACTOBACILLUS

The lactobacilli are perhaps the most heterogeneous group of LAB, and within such diversity the potential exists for equally disparate antagonistic peptides. A not uncommon trait of bacteriocins from *Lactobacillus* is a limited or narrow range of inhibitory activity, usually within the Lactobacillaceae. Another feature encountered in earlier studies involving the lactobacilli was an inability to link plasmid production with plasmid-encoded genes (Barefoot and Klaenhammer, 1983; McCormick and Savage, 1983; Joerger and Klaenhammer, 1986); however, Muriana and Klaenhammer (1987) were able to document plasmid involvement in *L. acidophilus* 88. Also, some isolates of lactobacilli are strong producers of hydrogen peroxide (Wheater et al., 1952). Along with lactic acid, these compounds can interfere when screening for authentic bacteriocin activity in lactobacilli.

Early surveys of strains of lactobacilli for bacteriocin activity were conducted by De Klerk and coworkers (De Klerk and Coetzee, 1961, 1967; De Klerk and Smit, 1967) and Upreti and Hinsdill (1973, 1975). Barefoot and Klaenhammer (1983) detected bacteriocin production in 42 of 52 strains of *L. acidophilus* examined. Bacteriocin producers have been well represented in the genus *Lactobacillus*. De Klerk and Coetzee (1961) originally screened the supernatants of 189 strains of lactobacilli for lysogeny; for a small percentage of strains, however, the antagonism was not caused by phage. Follow-up work (De Klerk and Coetzee, 1967) with 125 strains of *Lactobacillus fermenti* found 20% of these cultures to be bacteriocin-positive versus strains of *L. fermenti* and *L. acidophilus* when screened on agar.

The broad-spectrum antibiotic lactocidin was first discovered in *L. acidophilus* (Vincent et al., 1959); however, as pointed out by Klaenhammer (1988), a significant portion of the early work involved antagonistic substances that were not characterized very well. The activity spectrum of lactocidin included both Gram-negative and Gram-positive bacteria, but lactocidin has not been demonstrated in lactobacilli since its original citation.

Other antibiotic substances demonstrating a wide spectrum of activity have been detected in *Lactobacillus*. Two bacteriocins produced by strains of *L. acidophilus*, acidolin (Hamdan and Mikolajcik, 1974) and acidophilin (Shahani et al., 1977), have wide spectra of activity. In the case of an antibiotic substance from *L. bulgaricus*, a negative biuret test indicated the purified antagonistic compound was not a polypeptide (Abdel-Bar and Harris, 1984; Abdel-Bar et al., 1987). Ultraviolet absorption and mass spectra revealed an aromatic moiety with a molecular weight of approximately

700. This substance was active against *Pseudomonas fragi*. The compound examined by Abdel-Bar and coworkers has characteristics similar to reuterin, a low-molecular-weight, nonproteinaceous substance produced by *Lactobacillus reuteri* (Axelsson et al., 1988; unpublished manuscript, North Carolina State University, Raleigh, in Daeschel, 1989). Besides such Gram-positive varieties as *Clostridium*, *Staphylococcus*, and *Listeria*, reuterin was also active versus the Gram-negative enteric bacteria *Salmonella* and *Shigella* as well as *Candida* (yeast) and *Trypanosoma* (protozoan). Such an extremely wide range of effectiveness usually does not represent bacteriocin activity. Kabuki et al. (1997) studied a bacteriocin produced by *L. reuteri* LA6, reutericin 6. The 2.7-kDa peptide lacked lanthionine residues and featured 67% hydrophobic and polar neutral amino acids; a narrow spectrum of activity was suggested. However, reutericyclin isolated from *L. reuteri* LTH2584 is not a bacteriocin but a negatively charged, highly hydrophobic tetrameric acid derivative with a molecular weight of 349 (Ganzle et al., 2000). Reutericyclin was effective against Gram-positive bacteria but not Gram-negative bacteria. Resistant to proteolytic digestion, reutericyclin is about 20 times less potent than the bacteriocins curvacin A and sakacin P. For example, these two bacteriocins could inhibit strains of *L. sakei* at levels of 0.003 mg/L, whereas against its most sensitive indicator strain (*E. faecalis*) the level of inhibition by reutericyclin was about 0.05 mg/L.

Gilliland and Speck (1977) surveyed the antagonistic activity of *L. acidophilus* toward intestinal and foodborne pathogens that included *S. aureus*, enterotoxigenic *E. coli*, *Salmonella* Typhimurium, and *C. perfringens*. The presence or extent of bacteriocin activity was not specifically addressed, but it was estimated that antagonism was not directly related to acids produced and that hydrogen peroxide was only partially responsible for the inhibitory effect because it was evident under anaerobic conditions in a prerduced medium.

Evidence for a 5400-dalton peptide with a broad range of activity was described by Mehta et al. (1983). Although the method for measurement of antagonistic effect was not clear, *Salmonella* Typhi, *P. aeruginosa*, and *E. coli*, as well as *S. aureus* and several bacilli, were inhibited by this trypsin- and chymotrypsin-sensitive bacteriocin produced by *L. acidophilus* AC.

Bulgarican was named by Reddy et al. (1983). Growth of *L. bulgaricus* DDS 14 at 45°C for 48 hours in milk media with methanol-acetone extraction and silica gel chromatography of the supernatant resulted in inhibition of both Gram-positive and Gram-negative bacteria, including *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Sarcina lutea*, *S. aureus*, *P. aeruginosa*, *P. fluorescens*, and *Serratia marcescens*. There was no demonstrable antifungal activity. The best effect was at a pH of less than 4.5, with pH 2.2 optimum.

As noted earlier, many bacteriocins from lactobacilli inhibit only other lactobacilli; examples include lactacins B and F. These proteins resemble each other and are produced by *L. acidophilus*. Lactacin B as produced by *L. acidophilus* N2 was first characterized by Barefoot and Klaenhammer (1983). The protein formed aggregates of approximately 100,000 daltons. Purification showed the protein to actually be of 6000 to 6500 daltons (Barefoot and Klaenhammer, 1984). It was active against *L. bulgaricus*, *Lactobacillus leichmannii*, *Lactobacillus helveticus*, and *Lactobacillus lactis* and was bactericidal, sensitive to proteases, and heat-stable (100°C for 1 hour). Nonspecific adsorption was demonstrated by the adherence of lactacin B to sensitive and insensitive cells. Production of lactacin B appears to be chromosomally linked because *L. acidophilus* N2 does not contain plasmid DNA.

Lactacin F is produced by *L. acidophilus* 88 (Barefoot and Klaenhammer, 1983). Besides the lactacin B indicators, lactacin F also inhibits *Lactobacillus fermentum* 1750 and *S. faecalis* 19433 (Muriana and Klaenhammer, 1987). Lactacin F is heat-stable (121°C for 15 minutes) and sensitive to trypsin, proteinase K, ficin, and subtilisin, and its production is pH-dependent. As noted earlier, lactacin F is a plasmid-linked property of *L. acidophilus* 88 (Muriana and Klaenhammer, 1987). Bacteriocin production and immunity are associated with resident plasmids of 4 and 27 MD. Genetic analysis of transconjugants acquiring these phenotypes suggested the involvement of transient plasmid determinants capable of chromosomal integration — that is, the occurrence of detectable plasmids or the “plasmid state” may be the result of a defective or incomplete mobilization event.

Muriana and Klaenhammer (1991a) purified lactacin F. Electron microscopy demonstrated micelle-like globular particles of the native protein. Gel filtration of the bacteriocin suggested a large macromolecular complex with a molecular weight of 180,000; further purification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified a 2500-dalton peptide that showed a 474-fold increase in activity; however, a protein as large as 6200 daltons or 56 amino acids was revealed by amino acid composition analysis. A segment of 25 N-terminal amino acid residues was determined by sequence analyses that aided in the genetic cloning of the lactacin F structural gene (Muriana and Klaenhammer, 1991b).

Raccach et al. (1989) described the inhibition of *L. monocytogenes* by *L. acidophilus* strains NU-A and 88 (the latter strain producing lactacin F; Muriana and Klaenhammer, 1987). Inhibition was demonstrated in milk, in which the presence of *L. acidophilus* prevented the growth of *L. monocytogenes* to the levels it would normally reach in pure culture. The lactobacilli lowered the pH of the milk to 4.7 within 24 hours, indicating that acid was active in antibiosis, but it was shown that *L. acidophilus* 88 was bacteriocidal against *L. monocytogenes* OH in the agar test system.

In an examination of the antibacterial activity of *L. acidophilus* for intended use as a dietary adjunct, Fernandes et al. (1988) used bile salts added to MRS broth to simulate concentrations in the human intestinal tract. Nutrient content also significantly affected the antimicrobial activity of *L. acidophilus*.

Ferreira and Gilliland (1988) presented an example of the disadvantage of having a bacteriocin producer in a mixed-strain starter culture. When *L. acidophilus* NCFM, a mixed-strain culture, was grown at pH 6.0, a slow-growing, bacteriocin-producing variety was able to predominate by killing the other strain(s) in the culture. When a multiple starter culture is used (e.g., probiotics, fermented foods and beverages), it should be examined during its formulation for a lack of strain compatibility because of the production of a bacteriocin by one member that eliminates other strains.

Lactocin 27 and helveticin J are both produced by strains of *L. helveticus*. Lactocin 27, as produced by *L. helveticus* LP27, was originally described as a protein-lipopolysaccharide complex of over 200,000 Da (Upreti and Hinsdill, 1973), but refinement of technique defined a glycoprotein of approximately 12,400 Da (Upreti and Hinsdill, 1975). The formation of aggregates by bacteriocins produced by LAB appears to be a rather common occurrence.

The bacteriocin produced by *L. fermenti* 466 appears to be similar to lactocin 27 (De Klerk and Smit, 1967; Upreti and Hinsdill, 1973). Lactocin 27 is digested by trypsin and pronase, and its stability at high temperatures is exceptional. There is no demonstrable loss of activity after a 1-hour exposure to 100°C (Upreti and Hinsdill, 1973, 1975). Biochemically, the bacteriocin from *L. fermenti* 466 also appears as a heat-resistant macromolecular lipopolysaccharide-protein complex. Loss of activity results with removal of the carbohydrate component. It is also inactivated by exposure to trypsin and pepsin but is resistant to urea, lysozyme, and heat (96°C for 30 minutes).

Helveticin J also has a limited range of antagonistic activity, inhibiting some strains of *L. helveticus*, *L. bulgaricus*, and *L. lactis* (Joerger and Klaenhammer, 1986). It is sensitive to pronase, trypsin, pepsin, ficin, proteinase K, and subtilisin, as well as heat (100°C for 30 minutes). In culture supernatants helveticin J forms aggregates of over 300,000 daltons, but with purification a molecular weight of 37,000 was identified that retained functionality. Extensive genetic investigation led Joerger and Klaenhammer (1986) to conclude that, like lactocin 27, the genes for production and immunity of helveticin J reside on the bacterial chromosome.

L. plantarum is an industrially important variety of lactobacilli, especially in the manufacture of pickles and olives. Lactolin was the first documentation of an antagonistic protein produced by *L. plantarum* (Kodama, 1952). It was originally distinguished in that it differed from nisin and diplococcin. Work on bacteriocins from *L. plantarum* languished from 1952 until Andersson (1986) published the inhibition of *S. aureus* and spheroplasts of Gram-negative bacteria by a bacteriocin from *L. plantarum* 83. Since then research activity has increased in this area (Klaenhammer, 1988).

West and Warner (1988) evaluated bacteriocin production in six strains each of *L. plantarum* and *L. mesenteroides* and found bacteriocin activity in *L. plantarum* 1193. This bacteriocin was

sensitive to proteolytic enzymes, and its activity was reduced by lipase and α -amylase. It was antagonistic toward two other strains of *L. plantarum*, *L. mesenteroides* 8015, and *Pediococcus damnosus* 1832, but it was ineffective against single strains of *Leuconostoc cremoris*, *Lactobacillus casei*, *L. fermentum*, and *L. acidophilus*. The bacteriocin was not isolatable from liquid culture. The authors stated that a bacteriocin effective against clostridia would be most valuable in a *L. plantarum* starter culture used to make silage.

Plantaricin A, as produced by *L. plantarum* C-11, was reported by Daeschel et al. (1990). Their study showed a bacteriocin that was heat-stable (100°C for 30 minutes) and active over a pH range of 4.0 to 6.5 and had an approximate molecular weight of less than 8000 as based on retention in dialysis membranes. The spectrum of activity was limited to those LAB that *L. plantarum* C-11 would normally encounter in its function of fermenting cucumbers — that is, other *L. plantarum*, *P. pentosaceus*, and *Leuconostoc paramesenteroides* populations. A similar range of inhibition was found by Rammelsberg and Radler (1990) with lactobacilli isolated from plants or fermenting material. For the 15% of isolates that were found to be bacteriocin-positive, the range of effectiveness was primarily *Lactobacillus*, *Pediococcus*, and *Leuconostoc*.

Dry sausage was the source of *L. plantarum* UG1, producer of plantaricin UG1 (Enan et al., 1996). The thermostable peptide has a molecular weight of 3 and 10 kDa, and amylolytic enzymes reduce its activity. Plantaricin UG1 is chromosomally encoded in strain UG1. Whether killed or not, all Gram-positive bacteria absorbed the bacteriocin, whereas Gram-negative bacteria did not absorb it. Plantaricin UG1 inhibited more than LAB as is the case with plantaricin A. *L. monocytogenes*, *B. cereus*, *C. perfringens*, and *Clostridium sporogenes* were inhibited.

Plantaricin C19 is a pediocin-like bacteriocin produced by *L. plantarum* C19 originally isolated from cucumbers (Atrih et al., 2001). With a weight of 3845.3 Da (36 amino acids) as determined by mass spectroscopy, the peptide is antilisterial with a preponderance of hydrophobic and basic amino acids. Its absorption to cells of target *Listeria* was diminished by the presence of NaCl, KCl, MgSO₄, and CaCl₂ possibly as a result of competitive ion adsorption on the cell surface by the salts.

Plantaricin LP84 from *L. plantarum* NCIM 2084 was found to be bactericidal and lytic against both Gram-positive bacteria (*B. cereus*, *S. aureus*, and LAB) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*; Suma et al., 1998). It is a heat-stable peptide of 1 to 5 kDa; its ability to inactivate these two types of Gram-negative bacteria is quite interesting as a broad-spectrum preservative.

A lantibiotic, plantaricin C, was isolated from a dairy strain of *L. plantarum*. In its two-part, 27-amino acid sequence, Turner et al. (1999) found one dehydroalanine, one lanthionine, and three α -methyl-lanthionine residues. The first part of the molecule (six amino acids) confers a strong positive charge, whereas the larger second part contains a lanthionine bridge to stabilize a globular structure to the bulk of the molecule that allows insertion of the bacteriocin into the target membrane opening pores to cause leakage of cytoplasm from the bacterial cell.

Strains of *L. sake* have been found to possess bacteriocin activity. Schillinger and Lucke (1989) surveyed 221 strains of lactobacilli isolated from meats and found 19 strains of *L. sake*, 3 strains of *L. plantarum*, and 1 strain of *Lactobacillus curvatus* to inhibit other lactobacilli. Evaluation of the antimicrobial spectra of the supernatants suggested that the bacteriocins were not identical. A bacteriocin from *L. sake* Lb 706, termed sakacin A, was effective against *L. monocytogenes* strains 8732 and 17a. Another 4 strains of *L. sake* and 1 strain of *L. plantarum* proved antilisterial as well. Synthesis and immunity of sakacin A was linked to an 18-MDa plasmid. In mixed culture, it was found that once the bacteriocin-positive strain reached maximal cell density, cells of the bacteriocin-sensitive strain were killed.

Lactocin S was identified by Mortvedt and Nes (1990). The bacteriocin is produced by *L. sake* L45 isolated from a dry, sausage fermentation. Lactocin S was described as moderately heat-stable, sensitive to proteases, and active against members in the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc*. It has approximately 33 amino acid residues (Mortvedt et al., 1991). Initial investigation of the plasmid biology in *L. sake* L45 revealed instability of bacteriocin production and immunity (Mortvedt and Nes, 1990). Two plasmids of approximately 50 and 34 kb were

associated with these traits. It was found that isolates of *L. sake* L45 that were bacteriocin-negative (Bac-) and negative for immunity to lactocins (Imm-) had lost the 50-kb plasmid; however, restriction enzyme analyses of the 50-kb plasmids from Bac+Imm+ and Bac-Imm+ isolates revealed distinct differences suggestive of a rearrangement of DNA bases between the plasmids.

In a study examining the effectiveness of *L. sakei* CTC 494 as a potential starter culture for sausage fermentation, Leroy and de Vuyst (1999) found that sodium chloride and sodium nitrite in the meat mixture interfered with production of sakacin K by the lactobacilli. With *L. sakei* CTC494 and sakacin K, Hugas et al. (1998) demonstrated an antagonistic effect to different extents against *L. innocua* CTC1014 in meats over a range of 4°C to 30°C, initial pH range of 5.5 to 6.5 packaged with oxygen-permeable film, under vacuum and under a modified atmosphere (80% oxygen, 20% carbon dioxide). Vacuum-packaged poultry breasts and cooked pork and modified atmosphere-packaged raw minced pork demonstrated the greatest degree of inhibition of *L. innocua*. An immediate bactericidal effect was evident in every product and atmosphere was evaluated when sakacin K was added.

Ganzle et al. (1999b) used a dynamic model of the stomach and small intestine to evaluate the antagonistic effect of bacteriocin-producing *L. curvatus* LTH 1174 on *E. coli* LTH 1600 and *L. innocua* DSM20649. The model, kept at 37°C, consisted of four serial compartments simulating the stomach, duodenum, jejunum, and ileum; it could be disinfected with steam and the flexible walls allowed contractions duplicating peristaltic movements. Results indicated that the ability to produce bacteriocins was unlikely to contribute to colonization of the human intestine with LAB starter cultures. It was felt that because of the low bile tolerance of meat lactobacilli, it was unlikely that bacteriocin production was a significant factor in killing *E. coli* or *L. innocua* in the gut; however, bacteriocin-producing lactobacilli that are bile-resistant may be useful in controlling pathogens in the small intestine.

A system for heterologous expression using a bacteriocin-negative strain (Lb790) of *L. sake* was developed by Axelsson et al. (1998). In it, two plasmids introduced into Lb790 allowed various bacteriocins (sakacin P, pediocin PA-1, and piscicolin 61) to be produced at levels equal to or exceeding levels obtained with the corresponding wild-type cultures. Genes necessary for transcriptional activation of the sakacin A promoter, and export and processing of bacteriocin precursors, are contained on the first plasmid. The second plasmid has the sakacin A promoter fused to the structural and immunity genes for the bacteriocin.

Two-peptide bacteriocins are more frequently encountered. For example, Cuozzo et al. (2000) investigated lactocin 705, a class IIb bacteriocin from *L. casei* CRL 705. For activity, lactocin 705 was found to require the presence of both peptides, 705 α and 705 β , each containing 33 amino acids and encoded on a 35-kb plasmid. Similar DNA base sequences with other class II bacteriocins were found regarding immunity determinants and maturation and transport proteins.

Prior to the discovery of two forms of lactocin 705, Vignolo et al. (1998) evaluated the effectiveness that lactocin 705 inhibited *L. monocytogenes* when contained in a meat slurry. It was found that ingredients (sodium chloride and sodium nitrite, ascorbic acid, alginate, and sodium lactate) used in the meat cure protected *L. monocytogenes* from the effects of lactocin 705; sodium lactate neutralized the effects of lactocin 705 to the greatest extent.

CARNOBACTERIUM

Once a member of *Lactobacillus*, *Carnobacterium* is comprised of nonaciduric lactobacilli producing L(+) lactic acid from glucose (Collins et al., 1987). In the past, the carnobacteria were sometimes referred to as the meat lactobacilli. It includes what were formerly species of nonaciduric *Lactobacillus piscicola* and *Lactobacillus divergens* and other nonaciduric lactobacilli from poultry and vacuum-packaged meat. Bacteriocins produced by this genus appear quite diverse, although common types are distributed within the group.

Ahn and Stiles (1990) examined bacteriocin activity produced by *Carnobacterium piscicola* LV 17. Activity was found against closely related LAB, *Enterococcus* species, and a strain of *L. monocytogenes* but not Gram-negative bacteria. Cell death was dependent on contact of the bacteriocin with the cell membrane of the target organism. They found bacteriocin production and resistance to the bacteriocin residing on plasmids of 40 and 49 MD. It was postulated that two bacteriocins are produced because the inhibitor substances of mutant strains containing either the 40- or 49-MD plasmids have different antimicrobial spectra.

Carnocin UI49 is a bacteriocin from a strain of *Carnobacterium* isolated from fish. Stoffels et al. (1992) purified and characterized UI49. It is a protein with a molecular weight of 4635 as determined by mass spectrometry. Amino acid analysis suggests that carnocin UI49 belongs to the lantibiotic class of bacteriocins. Apparently, UI49 is distinct from the bacteriocins produced by *C. piscicola* LV17, which do not contain lantibiotic-like amino acids.

C. piscicola JG126 was isolated from spoiled ham and found to produce piscicolin 126 that is effective against *L. monocytogenes* and other Gram-positive bacteria (Jack et al., 1996). The peptide has a molecular weight of 4417 (44 amino acids), and sequence analysis showed it to be a member of class IIa with close resemblance to pediocin PA-1 and sakacin P. In trials in deviled ham paste, piscicolin 126 inhibited *L. monocytogenes* for 14 days at 10°C, proving itself (final concentration 2048 AU/g) superior to two commercial preservatives, ALTA™2341 (final concentration 1% or 2048 AU/g) and Nisaplin™ (400 IU/g). The ingredient declaration of ALTA™2341 (Quest International Bioproducts) states it is a fermentation by-product of an LAB that includes corn syrup solids, hydrolyzed yeast, and hydrolyzed vegetable protein. Antibacterial components are not specified.

By comparing DNA sequences in data banks and confirming results with PCR, Herbin et al. (1997) found carnocin CP51 from *C. piscicola* CP5 to share homologies with carnobacteriocin BM1, whereas carnocin CP52 and carnobacteriocin B2, both produced by *C. piscicola* LV17, were similar. Strains CP5 and LV17 were isolated from different foods: *C. piscicola* CP5 from soft cheese and *C. piscicola* LV17 from vacuum-packed meat.

Blom et al. (2001) examined carnocin H, a class II bacteriocin produced by *Carnobacterium* species 377. Carnocin H contained approximately 75 amino acids. Increasing levels of pH, indicator bacteria, sodium chloride, agar, and soybean oil impeded antagonism by the peptide. Produced during the late stationary growth phase, carnocin H inhibited the nine strains of *L. monocytogenes* examined. Its inhibitory spectrum differed from that of *C. piscicola* LV61 and *C. divergens* 750.

LEUCONOSTOC AND OTHER LACTIC ACID-ASSOCIATED BACTERIA

Leuconostoc is a genus of heterofermentative lactic organisms with important commercial applications in the manufacture of cultured dairy and vegetable products. Usually the inhibition of other organisms by *Leuconostoc* is attributed to the excretion of acetic and lactic acids by its metabolizing cells (Devoyod and Poullain, 1988). Orberg and Sandine (1984) found six strains of *Leuconostoc* to produce substances with antibacterial activity versus other leuconostocs and *Streptococcus lactis* strains. These effects were measured using the agar plate methods of Kekessy and Piguët (1970). One strain, *Leuconostoc* species P0184, inhibited *S. cremoris* U134 but not the nisin producer *S. lactis* ATCC 11454. In subsequent work, Tsai and Sandine (1987) demonstrated the capability of *Leuconostoc dextranicum* to produce nisin. A 17.5-MDa plasmid from *S. lactis* 7962 that encoded for nisin biosynthesis and sucrose fermentation was transferred by conjugation into *L. dextranicum* 181. Not only was nisin production detected in a transconjugant, but there was significant overproduction of the bacteriocin (enough for the transconjugant to be termed a supernisin producer). Such a phenomenon is of obvious interest in the optimization of fermentations for commercial bacteriocin production.

Branen et al. (1975) investigated the inhibition of pseudomonads by culture supernatants of *Leuconostoc citrovorum* and *Streptococcus diacetylactis*. Organic acids were believed to play a

more significant role in the antagonism of *Pseudomonas* by *L. citrovorum* than for *S. diacetylactis*. From growth of *S. diacetylactis* a small-molecular-weight, heat-stable protein was isolatable that possessed activity against *Pseudomonas* at pH 6.0.

An amylase-sensitive bacteriocin, leuconocin S, produced by a strain of *L. paramesenteroides* isolated from retail lamb was characterized by Lewus et al. (1992). Leuconocin S is active against *L. monocytogenes*, *S. aureus*, *Yersinia enterocolitica*, and some strains of *C. botulinum*. Leuconocin S is a glycoprotein sensitive to α -amylase, trypsin, α -chymotrypsin, protease, and proteinase K but not to lipase or treatment at 60° for 30 minutes. Apparently, the bacteriocin forms aggregates. Molecular weights of 2000 and 10,000 were determined from sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) of the supernatants from culture broths.

L. mesenteroides TA33a was found to produce three bacteriocins, each with a different spectrum of inhibition (Papathanasopoulos et al., 1997). After purification of the bacteriocins, it was discovered that leucocin A-TA33a was identical to leucocin A-UAL 187 (Fleury et al., 1996).

Leucocin B-TA33a (3466 Da) appeared similar to mesenterocin 52B (Revol-Junelles and Lefebvre, 1996) and dextransin 24 (Revol-Junelles et al., 1996) and leucocin C-TA33a was a novel compound of 4598 Da. A-T33a and C-T33a both were antilisterial. Papathanasopoulos et al. (1997) conducted a survey of other strains of *Leuconostoc* and *Weissella* for the occurrence of the three different bacteriocins and found it relatively widespread; strains of *L. carnosum*, *W. paramesenteroides*, and *L. gelidum* produced the three bacteriocins.

In an effort to optimize bacteriocin production during batch fermentation, Krier et al. (1998) used *L. mesenteroides* subspecies *mesenteroides* FR52 for synthesis of mesenterocins 52A and 52B to find that syntheses for both bacteriocins was stimulated by slow growth rates of the culture. Optimal temperature and pH for mesenterocin 52A was 20°C and pH 5.5, whereas for mesenterocin 52B optimal parameters were 25°C and 5.0. As a result, strain FR52 could be grown under different conditions to favor one bacteriocin over the other. Additionally, different production kinetics were exhibited by the two bacteriocins.

Leucocin H is another two-peptide bacteriocin (Blom et al., 1999). Synthesized by *Leuconostoc* species MF215B, the two peptides called leucocin H α and H β both contain 40 amino acids and inhibit *L. monocytogenes*, *B. cereus*, and *C. perfringens* among other Gram-positive bacteria. Blom et al. (1999) found the two peptides to share little sequence homology with each other, and no sequence homology could be found with any other bacteriocin as well. It was noted that the addition of soy oil to agar had no effect on the effectiveness of leucocin H.

Callewaert et al. (2000) tested *Enterococcus faecium* CCM 4231 and *E. faecium* RZS C13 as starter cultures in Spanish-style dry sausage fermentation against nonpathogenic *Listeria*. They found the bacteriocins produced by both strains to be inactive against LAB but effective against *L. innocua* and *L. ivanovi*. Results indicated that the enterococci were effective against *Listeria* species decreasing their counts after 1 week of fermentation by 1 to 3 log₁₀ CFU/g. The sensory quality of the sausages was judged normal.

Enterocin CRL35 from *E. faecium* CRL35 has effectiveness against herpes viruses. Wachsman et al. (1999) demonstrated that this antibacterial polypeptide (3.5 KDa), inhibitive of *L. plantarum* and Gram-positive pathogens, showed a 2-log reduction of viable thymidine-kinase positive and deficient strains of herpes simplex virus type 1 and 2 in Vero and BHK-21 cells. Viral multiplication was inhibited in a dose-dependent manner. The antiviral effect of enterocin CRL35 did not appear to be the result of direct inactivation, suggesting a different mechanism from human defensins for antiviral effect. The bacteriocin may affect the viral reduction of host protein synthesis, thus impairing virus replication.

Additional bacteriocins from *Enterococcus* include enterocin P, enterocin B, and enterocins L50A and L50B. Enterocin P from *E. faecium*, is strongly antilisterial, pediocin-like in sequence, stable to 121°C for 15 min, and also effective versus *C. botulinum* (Cintas et al., 1997). Enterocin B, from *E. faecium* T136, acts synergistically with enterocin A and closely resembles carnobacteriocin A (Casaus et al., 1997). As noted by others, combinations of bacteriocins, in this case

enterocins A and B, were highly bactericidal as compared to individual bacteriocins. For enterocins L50A and L50B from *E. faecium* L50, a similar synergism was demonstrated against target Gram-positive bacteria, although L50A and L50B did not resemble the pediocin family of bacteriocins, but rather staphylococcal cytolysins having a hemolytic activity (Cintas et al., 1998).

Use of bacteriocin-producing enterococci in cheese has been shown to inhibit histamine formation in Manchego cheese. In separate cheese-making experiments, Joosten and Nunez (1996) demonstrated that inclusion of two bacteriocin-producing enterococci or a nisin-producing strain of *L. lactis* was effective in preventing formation of histamines in the cheese by histamine-producing *Lactobacillus buchneri*. In control cheeses, levels of histamine reached 200 mg per kg of cheese after 4 months of ripening. Production of pediocin PA-1, bavaricin A, lactococcin A, and a bacteriocin from *E. faecalis* was found to be ineffective in preventing histamine production.

Another group of food-grade organisms that are sometimes classified with the traditional LAB are the bifidobacteria. These anaerobic, Gram-positive rods are known for their fastidious nature. The benefit derived from their presence in the human intestinal tract is also well established (Hughes and Hoover, 1991). One of the major benefits is the reduction of enteric pathogens in the intestinal tract by bifidobacteria. The bifidobacteria excrete high levels of acetate, lactate, and some formate from their metabolism of fermentable sugars, and this is the primary mechanism of inhibiting detrimental gut organisms. Of course, the production of bacteriocins is another ecologic weapon that many acidulating bacteria produce. Jao et al. (1978) suggested that the antibacterial activity of *Bifidobacterium bifidum* may be caused by the production of antibacterial substances in addition to acids. Anand et al. (1985) detected potential bacteriocin activity in strains of *B. bifidum*. Cultures of both Gram-positive and Gram-negative bacteria were inhibited by growth supernatants, but the effect was absent above pH 4.5, and fractionated metabolites singly or in combination did not show antagonistic activity. Meghrouh et al. (1990) partially characterized a bacteriocin from an unspecified strain of *Bifidobacterium*. Susceptible to trypsin, pepsin, and pronase and functional between pH 2.0 and 10.0, the activity of the bacteriocin remained after 121°C for 15 minutes against lactococci, *Streptococcus salivarius* subspecies *thermophilus*, *L. acidophilus*, *Clostridium tyrobutyricum*, and *Clostridium butyricum*. No Gram-negative varieties were inhibited. In later work, Yildirim and Johnson (1998) screened for bacteriocin production in MRS broth from growth of five strains of *B. bifidum* and found only one strain (NCFB 1454) capable of producing a detectable bacteriocin. It was a proteinaceous compound of 3.3 kDa with resistance to exposure to 121°C for 15 minutes as well as pH extremes of 2.0 and 10.0. Designated bifidocin B, it was active against most strains from species representing *Listeria*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*.

Propionibacterium freudenreichii subspecies *shermanii* is used to produce a preservative ingredient called MicroGARD™. It is produced by pasteurization of propionibacteria-fermented skim milk and is estimated to be used in about one third of all cottage cheese made in the United States because of its ability to inhibit psychrotrophic spoilage bacteria (Daeschel, 1989). Its effectiveness is directed against most Gram-negative bacteria and some fungi but not Gram-positive bacteria. Although propionic acid, diacetyl, acetic acid, and lactic acid are present, the most active component of MicroGARD is a heat-stable peptide of about 700 Da that is inactivated by proteolytic enzymes (N. Al-Zoreky, M.S. thesis, Oregon State University, Corvallis, 1988).

Evidence of a bacteriocin produced by *Propionibacterium thoenii* P127 was shown by Lyon and Glatz (1991). The bacteriocin, called propionicin PLG-1, was found to be heat-labile (85°C for 30 minutes), sensitive to several proteolytic enzymes, stable at pH 3.0 to 9.0 (greatest activity is at pH 7.0), and effective against a wide range of Gram-positive and Gram-negative bacteria, as well as some yeasts and molds. As measured by well diffusion assays, activity was discovered versus all propionibacteria, except for strains of *P. freudenreichii* subspecies *freudenreichii* and *P. freudnerichii* subspecies *shermanii*. LAB were inhibited (lactococci, lactobacilli, and pediococci) but representative strains of clostridia, bacilli, *S. aureus*, and *S. faecalis* were not. For Gram-negative bacteria, pseudomonads were significantly inhibited, but cultures of salmonellae, *A. hydrophila*, and

Y. enterocolitica were not. Fungistatic rather than fungicidal activity was found against some *Saccharomyces* strains and *Candida lipolytica*. *Aspergillus wentii* and seven other fungi were inhibited. Distinct from MicroGARD, propionicin PLG-1 demonstrated two molecular weights indicative of different protein aggregate forms. Gel-filtration studies determined complexes of approximately 10,000 Da and of more than 150,000 Da, with further purification revealing activity specific to the 10,000-Da molecule. The authors suggested a parallel relationship of propionicin PLG-1 with lactacin B as described by Barefoot and Klaenhammer (1983), in that both bacteriocins were first isolated as large aggregates that required SDS-PAGE purification into their monomeric forms. The need was also stated for a defined growth medium free of contaminating proteins that associate with bacteriocins to hinder production and purification. Detection and production of propionicin PLG-1 was improved by Hsieh et al. (1996) who increased the sensitivity of a standard well-diffusion assay. The assay was optimized by use of wells of specific dimensions into a solid medium containing 2.5% agar, 0.85% NaCl, and 0.1% Tween 80. The use of 0.1% Tween 80 in the buffer used for dialysis and resuspension of precipitated protein also improved purification of the bacteriocin. The preferred medium for growth of *P. thoenii* P127 for production of PLG-1 was 3:1 beet molasses:corn steep liquor, whose yield was five times higher than sodium lactate broth.

Jensenin G is a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Grinstead and Barefoot (1992) found that *P. jensenii* P126 inhibited closely related varieties of dairy propionibacteria, as well as species of *Lactobacillus* and *Lactococcus*, under anaerobic conditions. The bacteriocin was inactivated by pronase E, proteinase K, and type 14 protease but stable to freezing, cold storage (4°C for 3 days), and heat treatment (100°C for 15 minutes). The stability of jensenin G differs from the lability of propionicin PLG-1 to 85°C. The lack of detectable plasmids in *P. jensenii* P126 suggests a chromosomal location for the gene(s) of jensenin G.

Faye et al. (2000) characterized propionicin T1 at the molecular level. It was isolated from two different strains of *P. thoenii* and showed no sequence similarity to other bacteriocins. Propionicin T1 inhibited most of the propionibacteria tested as well as a strain of *L. sake*. Evidence was lacking that T1 possesses a wider target range. The mature form of the bacteriocin had 65 amino acids. It was noted that both producers of propionicin T1 were sensitive to it as well, suggesting that the immunity factors may not be constitutively expressed but instead may be regulated to production of T1. Cells early in the growth cycle seemed sensitive to the bacteriocin that is normally produced in late logarithmic stage.

BACTERIOCINS IN PACKAGING FILM

Incorporation of bacteriocins into packaging films has been examined. Coma et al. (2001) incorporated nisin into edible cellulosic films made with hydroxypropylmethylcellulose by adding nisin into the film-forming solution. Inhibitory effect could be demonstrated against *L. innocua* and *S. aureus*, but film additives such as stearic acid used to improve the water vapor barrier properties of the film significantly reduced inhibitory activity. It was noted that desorption from the film and diffusion into the food required further optimization for nisin to function more effectively as a preservative agent in the packaged food. *L. innocua* and *S. aureus* (along with an indicator strain of *L. lactis* subspecies *lactis*) were also used in a study by Scannel et al. (2000) of cellulose-based bioactive inserts and antimicrobial polyethylene/polyamide pouches. Lactacin 3147 and nisin were the tested bacteriocins. Although lactacin 3147 adhered poorly to plastic film, nisin bound well and the bioactive film made with nisin was stable for 3 months with or without refrigeration. Bacterial reductions of up to 2 log₁₀ CFU/g in vacuum-packed cheese were seen in combination with modified atmosphere packaging (MAP) with storage at refrigeration temperatures. Cellulose-based bioactive inserts were placed between sliced products of cheese and ham under MAP. Inserts with immobilized nisin reduced *L. innocua* by >3 log₁₀ CFU/g in cheese after 5 days at 4°C and by approximately 1.5 log₁₀ CFU/g in sliced ham after 12 days, whereas *S. aureus* was reduced by 1.5 and 2.8 log₁₀ CFU/g in cheese and ham, respectively. Although shelf-life was extended in these products as

TABLE 13.1
Requirements for Use of Naturally Occurring Antimicrobial Substances as Food Preservatives

Toxicology must be acceptable by recognized authorities.

It must not have any deleterious effect on any of the organoleptic properties of the food in which it is used.

Its cost of use must be economically acceptable to the industry.

It must be stable during storage before use, and if its action depends on a residual, it must be sufficiently stable for the shelf life of the food.

It should be effective at relatively low concentrations.

It should have no medical use.

Source: Adapted from Heinemann et al. (1965) by Daeschel (1989).

populations of LAB were reduced, the primary thrust was toward control of specific anticipated pathogens in the product. In this regard, Rhodia, Inc. is developing a casing to be used in hot dog manufacture and other cooked meats (D. Willrett, personal communication, 2002). The film harbors a proprietary combination of bacteriocins, enzymes, and botanicals. The components have received regulatory clearance. The approach is to cook the meat product while tightly contained within the bioactive casing or wrapper. The target is *L. monocytogenes*, and results are described as very promising. The added cost is considered economically sound given the large product recalls experienced by major meat brands as the result of product contamination with *L. monocytogenes*.

CLOSING REMARKS

Hechard et al. (1990) surveyed goats' milk for bacteriocin-like activity against *L. monocytogenes* and *S. aureus*; screenings of LAB isolates such as these from food sources will certainly continue as the search for valuable new peptides draws interest (Harris et al., 1989; Okereke and Montville, 1991; Lewus et al., 1991; Roller, 1995; Janes et al., 1999). Although the production of bacteriocins by pathogens effective against other pathogens is well documented (Tagg et al., 1976), it is doubtful that even with confirmed safety and purity, bacteriocins, such as monocins of *L. monocytogenes*, botocins of *C. botulinum*, or staphylococcins of *S. aureus*, would be approved for food use. Use of these peptides or proteins would bear the stigma and concern related to their site of production.

Other than nisin, no other bacteriocin has been approved for use as a preservative compound in the United States. At this writing, no other bacteriocin besides nisin has met the criteria as a cost-effective, suitable antimicrobial additive ready to be petitioned to the FDA for food use (Table 13.1). One possible concern is that when a bacteriocin is used as a food additive, it may far exceed the concentration to which a consumer is normally exposed by ingestion of fermented foods containing bacteriocin-producing cultures. Such high levels may trigger an allergic or toxicologic response, although this is believed highly unlikely. If bacteriocins of LAB are added at levels equivalent to that found in cultured foods, then they should be safe for consumption (Gorris, 1997); however, toxicologic testing is required and costs can be daunting.

Adding to the issue of costs is the required levels of bacteriocin necessary in the food to demonstrate effectiveness over the shelf-life of the product. Efficacy testing continues over a range of different, individual products; examples include optimization of enterocin CCM 4231 versus *L. monocytogenes* in dry fermented salami (Laukova et al., 1999); development of a lacticin 3147-enriched whey powder against *L. monocytogenes* and *S. aureus* in dairy-related products (Morgan et al., 1999); and improved control of *L. monocytogenes* with semipurified bacteriocins from *Carnobacterium* species in vacuum-packed, refrigerated cold-smoked salmon (Duffes et al., 1999). Studies such as these indicate that necessary bacteriocin concentrations can be quite high and issues of stability and consistency of effectiveness in the product remain, as do problems of low yield in

production of purified or semipurified preparations. Fermentation optimization is necessary to lower costs. As a result of the inherent difficulties with purified bacteriocin preparations, use of bacteriocin-producing cultures of LAB in food fermentations or incorporation of food ingredients containing bacteriocin-containing growth extracts (e.g., MicroGARD™ and ALTA™2341; Hugas, 1998) are alternative approaches that will continue to be used in those situations and products where these procedures or approaches have merit. Modification of starter cultures used in commercial fermentations with genes for bacteriocin production and immunity can be expected to continue (Ross et al., 1999; Lucke, 2000).

As noted by Konings et al. (2000), elucidation of signaling mechanisms for synthesis of bacteriocins along with a better understanding of the mode of action of bacteriocins should lead to expanded applications for these peptides. Clearly similar research thrusts in these and related areas will steadily evolve to increase our knowledge base and provide answers to address the questions limiting use of bacteriocins. Additionally, it can be assumed that nisin and other bacteriocins will continue to be incorporated and developed into hurdle concept methods and processes of food preservation for individual products. Use of bacteriocins of LAB to specifically inhibit pathogens such as *L. monocytogenes* and *S. aureus* can expect attention to improve food safety. It may be possible to target Gram-negative bacteria in foods by inserting genes from bacteriocins from Gram-negative bacteria into fermentative Gram-positive bacteria (Stiles, 1996; Hugas, 1998).

Protein engineering of bacteriocins can be expected to persist and possibly thrive. For example, Rollema et al. (1995) improved the solubility and stability of nisin Z by replacing Asn-27 or His-31 with lysine; Johnsen et al. (2000) improved the stability of pediocin PA-1 at 4°C and room temperature by replacing Met-31 with alanine, isoleucine, or leucine. Examples such as these indicate solutions to some of the problems related to bacteriocin application.

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14 Naturally Occurring Compounds – Plant Sources

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CONTENTS

Introduction	429
Sources of Natural Antimicrobials from Plants	431
Testing the Efficacy of Antimicrobials	432
Natural Phenolic Compounds	435
Other Plants as Sources of Antimicrobial Agents	439
Mechanisms of Action	440
Factors Affecting Activity	441
Application in Foods	442
Final Remarks	444
Acknowledgments	446
References	446

INTRODUCTION

Food market trends are changing. Consumers demand more high-quality foods with freshlike attributes (Gould, 1995a, 1995b, 1996); consequently less extreme treatments and/or additives are being required. To satisfy consumer demands, adjustments or reductions in conventionally used preservation techniques must be accomplished. Gould (1992, 1995a) identified some food characteristics that must be attained in response to consumer demands; most of them occur within minimal processing concepts. Attributes include less heat and chill damage; fresh appearance; less acid; and low in additives, salt, sugar, and fat. These possible changes have important and significant implications from a microbiological point of view. To satisfy market requirements, the safety and quality of foods have to be based on substantial improvements in traditional preservation methods or on the use of emerging technologies (Gould, 2002).

Consequences of microbial growth in foods include consumer hazards as a result of the presence of pathogenic organisms or microbial toxins, as well as economic losses as a result of spoilage (Davidson, 2001). Inactivation, growth delay, or growth prevention of spoilage and pathogenic microorganisms are main objectives of food preservation. Several factors influence microbial growth and survival; appropriate modification and/or application of these factors is the base of preservation technologies. Food preservation technologies protect foods from the effects of microorganisms and inherent deterioration. Major food preservation technologies can be classified as those that act mainly by preventing or slowing down microbial growth (low temperature, reduced water activity, acidification, fermentation, modified atmosphere packaging, addition of antimicrobials, compartmentalization in water-in-oil emulsions), those that act by inactivating microorganisms (heat pas-

teurization and sterilization, microwave heating, ionizing radiation, high pressure, pulsed electric fields, high frequency ultrasound), and those that prevent or minimize entry of microorganisms into food or remove them (aseptic handling or packaging, centrifugation, filtration) (Gould, 1995, 2002; Sofos et al., 1998). In addition, techniques in combination, based on the hurdle technology concept (Leistner, 1995, 2000), may act by inhibiting or inactivating microorganisms, depending on the combination of hurdles applied to achieve food preservation (López-Malo et al., 2000).

The use of chemical agents exhibiting antimicrobial activity (by inhibiting and/or reducing microbial growth or even by inactivating undesirable microorganisms) is one of the oldest and most traditional food preservation techniques. Antimicrobial agents are chemical compounds added to, or present in, foods that retard microbial growth or cause microbial death. As early as 8000 BC, people did preserve the excess meat and fish by smoking and dry salting; during 6000 to 1000 BC, sulfur dioxide by burning sulfur was used to sanitize fermentation and storage equipment in the production of wine in Egypt (Ray and Daeschel, 1992; López-Malo et al., 2000). Antimicrobial activities of several plants used today as seasoning agents in foods and beverages have been recognized for centuries. The early Egyptians used plant extracts (spices and oils) as antimicrobials for the preservation of food as well as for embalming. Although ancient civilizations recognized the antiseptic or antimicrobial potential of many plant extracts; it was not until the 18th century that scientific information established the preservative effects of several plants.

Concerns about the use of antimicrobial agents in food products have been discussed for decades (Parish and Carroll, 1988). Both the increasing demand for reduced-additive (including antimicrobial agents) and more “natural” foods and the increasing demand for greater convenience have promoted the search for alternative antimicrobial agents or combinations to be used by the food industry (López-Malo et al., 2000). In this search, a wide range of natural systems from animals, plants, and microorganisms is being studied (Beuchat and Golden, 1989; Board, 1995; Nychas, 1995; Hill, 1995; Sofos et al., 1998; López-Malo et al., 2000; Davidson, 2001; Gould, 2002; Chikindas and Montville, 2002). However, mainly economic aspects originated in the strict requirements to obtain approval and efforts to get the product onto the market restrict the spectrum of new chemical compounds that can help in the preservation of foods; furthermore, the approval process is very long (10 to 12 years). The application of chemical antimicrobial agents in food preservation is regulated in the United States by the Food and Drug Administration (FDA) and in other countries by appropriate corresponding authorities (Sofos and Busta, 1992). The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations, testing and recommending usage and safety of chemicals in foods and average daily intakes (ADI), regulate chemical antimicrobials internationally. In the United States, chemicals in foods are examined according to the Food Additives Amendment of the Food, Drug and Cosmetic Act, specifying the procedures and conditions required for a chemical food additive to be approved. These obstacles have originated the search of emerging preservatives by examining compounds already used in the food industry, perhaps with other purposes, but with potential as antimicrobials, approved and not toxic in the used levels; many of them are classified as generally recognized as safe (GRAS). Within these compounds are, for example, the so-called “green chemicals” present in plants that are used as flavor ingredients.

Naturally occurring antimicrobial compounds are abundant in the environment. Major antimicrobial systems naturally present in plants and animals or produced by microorganisms or that are considered within this classification are shown in Table 14.1. Some of these natural antimicrobial systems are already used for food preservation, whereas many others are just being studied to be used in foods (Gould, 1995). Development, exploration, and use of naturally occurring antimicrobials in foods; chemistry and food safety/toxicity aspects; antimicrobial activity; and mechanisms of action are covered in excellent reviews by Wilkins and Board (1989), Zaika (1988), Shelef (1983), Conner (1993), Nychas (1995), Beuchat and Golden (1989), Branen et al. (1980), Board (1995), Smid and Gorris (1999), López-Malo et al. (2000), Davidson (2001), and Gould (2002). Sofos et al. (1998) prepared a review following a recommendation by the Council for Agricultural

TABLE 14.1
Major Natural Antimicrobial Systems

Origin	Antimicrobial Agent	Examples	
Animals—constitutive	Lactoperoxidase, lactoferrin	Milk	
	Lysozyme, ovotransferrin, avidin	Eggs	
	Transferrins	Serum	
	Myeloperoxidase	Phagosomes	
Animals—inducible	Antibodies	Immune system	
	Attacins, cecropins	Insects	
Microorganisms	Nisin, pediocin, other bacteriocins	Lactic acid bacteria	
	Pimaricin, subtilin, natamycin, diacetyl	Other microorganisms	
Plants—preinfectious	Organic acids	Herbs, spices, and other plants	
	Phenolic compounds		
	Methylated flavones		
	Flavonols		
	Alkaloids (e.g., α -tomatine)		
	Hydroxyphenyl—threne derivatives		Lactones
	Protein-like compounds		
	Glucosides, glycosides, dienes		
Plant—Postinfectious	Phenolic compounds	Infected or injured plants	
	Sulfoxides		
	Isothiocyanates		
	Phytoalexins		

Source: Adapted from Gould, 1995; Wilkins and Board, 1989; Beuchat and Golden, 1989; Nychas, 1995; Hill, 1995; and Smid and Gorris, 1999.

Science and Technology of the United States. Although many natural systems have potential to be used as antimicrobials, this chapter will focus mainly on natural antimicrobials from plants and their possible application into foods.

SOURCES OF NATURAL ANTIMICROBIALS FROM PLANTS

Plants, herbs, and spices as well as their derived essential oils and isolated compounds contain a large number of substances that are known to inhibit various metabolic activities of bacteria, yeast, and molds, although many of them are yet incompletely exploited. Wilkins and Board (1989) reported that more than 1340 plants are known to be potential sources of antimicrobial compounds; about 60 are mentioned by Nychas (1995) and Beuchat (1994). The antimicrobial compounds in plant materials are commonly contained in the essential oil fraction of leaves (rosemary, sage), flowers and flower buds (clove), bulbs (garlic, onion), rhizomes (asafoetida), fruit (pepper, cardamom), or other parts of the plant (Nychas, 1995; Shelef, 1983). Table 14.2 presents a list of some of the more highly recognized plants, herbs, and spices that have been reported as sources of natural antimicrobials. These compounds may be lethal to microbial cells or they may simply inhibit the production of a metabolite (e.g., mycotoxins) (Beuchat, 1994; Davidson, 2001). Zaika (1988) reviewed the literature reporting the antimicrobial activity of many spices and classified their inhibitory activities as strong, medium, or weak. According to this ranking, cinnamon and clove were listed as exhibiting a strong inhibitory effect, and allspice was classified with a medium inhibitory effect. Conner and Beuchat (1984a) screened 32 essential oils from plant sources for inhibitory effects on 13 food spoilage and industrial yeasts and identified cinnamon, allspice, and clove among the most inhibitory tested. Antimicrobial activity of cinnamon, allspice, and cloves

TABLE 14.2
Selected Plants and Their Major Antimicrobial Compounds

Plant (Scientific Name)	Major Components (in descending order)
Allspice (<i>Pimenta dioica</i>)	Eugenol, methyl ether cineol
Basil (<i>Ocimum basilicum</i>)	d-linalool, methyl chavicol, eugenol, cineol, geraniol
Black pepper (<i>Piper nigrum</i>)	Monoterpenes, sesquiterpenes
Bay (<i>Laurus nobilis</i>)	Cineol, l-linalool, eugenol, geraniol
Caraway seed (<i>Carum carvi</i>)	Carvone, limonene
Celery seed (<i>Apium graveolens</i>)	d-limonene
Cinnamon (<i>Cinnamomum zeylanicum</i>)	Cinnamic aldehyde, l-linalool, p-cymene, eugenol
Clove (<i>Syzygium aromaticum</i>)	Eugenol, cariofilene
Coriander (<i>Coriandrum sativum</i>)	d-linalool, d- α -pinene, β -pinene
Cumin (<i>Cuminum cyminum</i>)	Cuminaldehyde, p-cymene
Fennel (<i>Foeniculum vulgare</i>)	Anethole
Garlic (<i>Allium sativum</i>)	Diallyl disulfide, diethyl sulfide, diallyl trisulfide, allicin
Lemongrass (<i>Cymbopogon citratus</i>)	Citral, geraniol
Marjoram (<i>Origanum majorana</i>)	Linalool, cineol, methyl chavicol, eugenol, terpineneol
Mustard (<i>Brassica hirta</i> , <i>B. juncea</i> , <i>B. nigra</i>)	Allyl-isothiocyanate
Onion (<i>Allium cepa</i>)	d-n-propyl disulfide, methyl-n-propyl disulfide
Oregano (<i>Origanum vulgare</i>)	Thymol, carvacrol, α -pinene, p-cymene
Parsley (<i>Petroselinum crispum</i>)	α -pinene, fenol-eter-apiol
Rosemary (<i>Rosmarinus officinalis</i>)	Borneol, cineol, camphor, α -pinene, bornyl acetate
Sage (<i>Salvia officinalis</i>)	Thujone, cineol, borneol, thymol, eugenol
Tarragon (<i>Artemisia dracunculus</i>)	Methyl chavicol, anethole
Thyme (<i>Thymus vulgaris</i>)	Thymol, carvacrol, l-linalool, geraniol, p-cymene
Vanilla (<i>Vanilla planifolia</i> , <i>V. pompona</i> , <i>V. tahilensis</i>)	Vanillin, vanillic, p-hydroxybenzoic, p-coumaric acids

Source: Adapted from López-Malo et al., 2000; Davidson and Naidu, 2000; Shelef, 1983; Farrell, 1990.

is attributed to eugenol (2-methoxy-4-allyl phenol) and cinnamic aldehyde, which are major constituents of the volatile oils of these spices. Cinnamon contains 0.5% to 1.0% volatile oil, which contains 65% to 75% cinnamic aldehyde and 8% eugenol. Allspice contains up to 4.5% volatile oil, of which 80% is eugenol. Clove buds have an average essential oil content of 17% that is 93% to 95% eugenol (Bullerman et al.; 1977; Farrell, 1990).

Major components with antimicrobial activity found in plants, herbs, and spices are phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids. As a rule it has been reported that antimicrobial activity of essential oils depends on the chemical structure of their components and on their concentration. Shelef (1983) mentioned that simple and complex derivatives of phenol are main antimicrobial compounds in essential oils from spices. Katayama and Nagai (1960) recognized eugenol, carvacrol, thymol, and vanillin as active antimicrobial compounds from plant essential oils. Aliphatic alcohols and phenolics were also reported as fungal growth inhibitors by Farag et al. (1989). Chemical structures of selected antimicrobial compounds from plant origin are presented in Figures 14.1 and 14.2.

TESTING THE EFFICACY OF ANTIMICROBIALS

Essential oils of a large number of plants possess useful biological and therapeutic activities and are extensively used in the preparation of pharmacologic drugs. They are commercially recovered from plant materials primarily by steam distillation, solvent extraction, or pressing, and their use in the food industry is influenced by the nature of their constituents. Antimicrobial activity of essential oils and extracts from plants, herbs, and spices depends on the extraction method but also

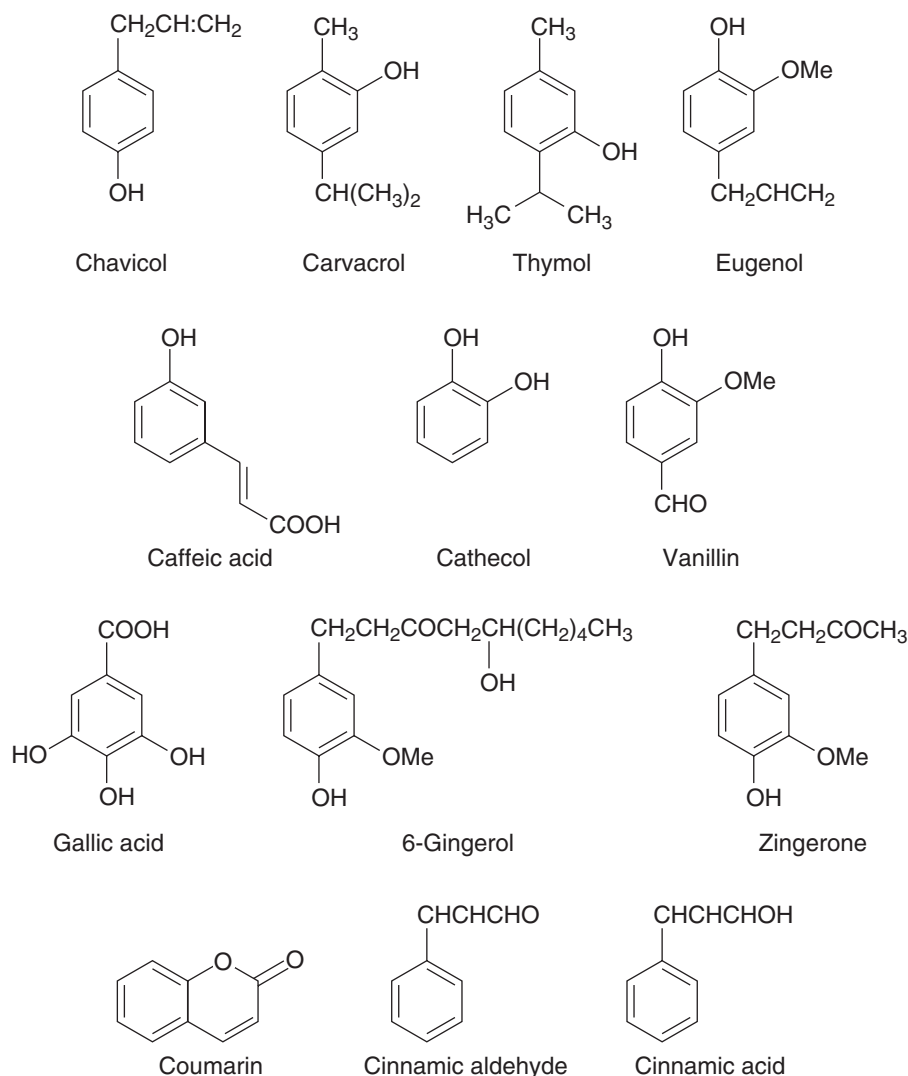


FIGURE 14.1 Selected naturally occurring plant compounds with antimicrobial activity.

on the initial quantity of essential oil in the plant. Within the same spice or plant, the levels of constituents and therefore active antimicrobial groups can substantially vary. Also, geographic zone of cultivation may influence extract composition. Antimicrobial effectiveness of lemon grass essential oil varied depending on the harvesting time within the year (Mishra and Dubey, 1994). During May to December it was more effective inhibiting 100% of the evaluated microbial strains, but the essential oil from plants collected during February to April was only 73% to 80% effective. Therefore, there is a necessity to establish methods to fix or standardize essential oil purity or concentration of active components. Knowledge of the qualitative and quantitative composition of essential oils will allow obtaining valuable data in systematic biological studies of plants as antimicrobial agents. Differences in antimicrobial effectiveness of plant extract compounds from different sources, extraction methods, and geographic zones can be diminished if the active antimicrobial compounds from extracts or essential oils are identified. Adoption of plant or spice essential oils as alternatives to other preservatives will depend on antimicrobial uniformity and/or on the analytic methods available to normalize their antimicrobial potency (López-Malo et al.,

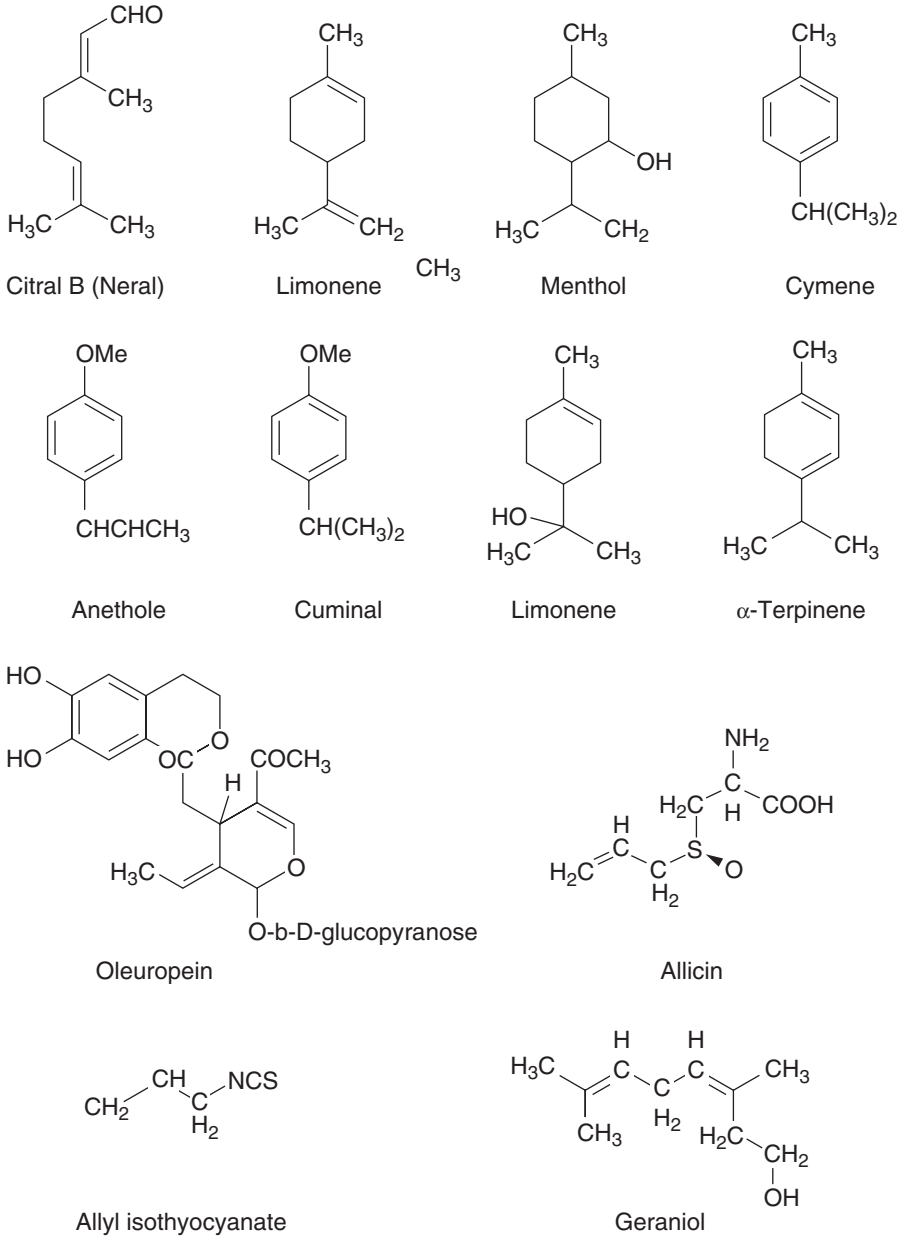


FIGURE 14.2 Selected naturally occurring plant compounds with antimicrobial activity.

2000). The concentration of antimicrobial compounds varies depending on the type of plant; among the richest are clove, nutmeg, and laurel, and the major constituents in most cases are phenolic compounds. Table 14.2 presents major and minor antimicrobials constituents of some plant extracts.

Many publications about the antimicrobial activity of extracts, oils, spice and herbs can be found in the food-related scientific literature. However, it is difficult to make quantitative comparisons of their effects at least partially because of the great variety of methods used to evaluate antimicrobial efficiency (Zaika, 1988; Davidson and Parish, 1989). Zaika (1988), Davidson and Parish (1989), and Parish and Davidson (1993) have critically discussed the numerous tests that have been used as well as the factors that are important to be considered in determining the

effectiveness of natural antimicrobials in foods. Screening and isolation of antimicrobial agents from plants require a multidisciplinary approach (Davidson and Naidu, 2000). *In vitro* or *in vivo* screening bioassays are useful determining factors for successful isolation of active compounds. Solvent selection for extraction including solvent ability to extract components is an important factor determining operation efficiency. Ghisalberti (1993) reviewed several techniques to isolate antimicrobial active components from plant sources. Dry plants could be extracted with a variety of solvents and sometimes sequentially from low to high polarity. Polar solvents such as ethyl acetate or methanol are often used. Ethyl acetate theoretically only extracts by leaching the sample, and alcoholic solvents presumably rupture cell structures (membranes) extracting also intracellular materials. For fresh plant materials (with high water content) a solvent mixture of dichloro-methane-methanol gave better results during extraction. Methanol separation and sample partition followed by ethyl acetate and butanol extraction help to separate lipophilic compounds from water-soluble materials.

Because many of the constituents of the extracts and evaluated oils are phenols, the phenolic coefficient technique is one of the endpoint methods for testing disinfectants that has been used to quantify their effects. This term, proposed by Martindale (1910), is expressed as the ratio between minimal concentrations of phenol and evaluated substance that inhibits a specific microorganism for a given incubation time. In this test, one of the microorganisms approved as reference (*Salmonella* Typhi, *Staphylococcus aureus*, or *Pseudomonas aeruginosa*) is exposed to several dilutions of the compound and phenol by 5, 10, and 15 minutes. The microorganism is plated in an antimicrobial-free media, and the phenolic coefficient is calculated with the dilutions that inhibit microbial growth in a 10-minute treatment and with the ones that do not inhibit it after 5 minutes (Martindale, 1910). Constituents of essential oils of oregano, thyme, cinnamon, and clove were found to be much more active than phenol, with phenolic coefficients greater than 1 (Martindale, 1910). Müller (1981) reported phenolic coefficients of 0.4, 10.0, 7.1, 5.4, 14.0, 0.4, and 13.4 for essential oils of anise, cardamom, cinnamon, coriander, fennel, lime, and thyme, respectively. Essential oils with high phenolic coefficients would have potential as antimicrobial agents at least for the bacteria used as reference microorganisms.

In vitro or explanatory (endpoint and descriptive methods) and applied (inhibition curves and endpoint methods) tests are the most used methods for efficiency evaluation of essential oils of plant and spices. A great number of these studies have been accomplished *in vitro*, and only few of them have been accomplished in foods. Within the evaluation methods in model systems, descriptive (inhibition curves) methods have been commonly used for bacteria and yeasts, while final point tests have been used for bacteria, yeast, and molds. Zaika (1988) concluded that many factors affect antimicrobial activity of spices, extracts, and essential oils and mentioned that several aspects must be considered and reported. The observed inhibition depends on the evaluation method. Microorganisms differ in their resistance toward a spice or herb and a given microorganism differs in its resistance to different spices or herbs. Food components can affect increasing (by the presence of acids, humectants, antimicrobials, etc.) or reducing (by partitioning of active components into the lipid phase, etc.) the antimicrobial capacity. Antimicrobial efficiency of a spice or herb depends on its origin, handling, processing, and storage.

NATURAL PHENOLIC COMPOUNDS

Derivatives of phenol, called phenolics, contain a phenol molecule with one or more substitutes. This chemical alteration of phenol may increase its antimicrobial activity. Phenolic compounds have been used as antimicrobial agents since the early use of phenol as sanitizer in 1867 (Davidson, 1993). As food antimicrobials, phenolic compounds can be classified, following Davidson (1993), as those currently approved (parabens), those approved for other uses (antioxidants), and those found in nature (polyphenolics, phenol). Parabens (methyl, propyl, and heptyl esters of p-hydroxybenzoic acid) are allowed in many countries as food antimicrobials for direct usage. Phenolic

antioxidants, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) are approved as food antioxidants to prevent rancidity in fats, oils, and lipid foods (Sahidi et al., 1992). BHA, BHT, and TBHQ are also recognized as compounds that have antimicrobial activity against bacteria, fungi, viruses, and protozoa (Branen et al., 1980). Ward and Ward (1967) were the first to report the antimicrobial activity of synthetic phenolic antioxidants. Naturally occurring phenolic compounds are widespread in plants and may be found in a great variety of food systems, and as phenol derivatives they may have antimicrobial activity. These naturally occurring phenols and phenolic compounds may be classified into the following groups: simple phenols and phenolic acids (e.g., p-cresol, 3-ethylphenol, vanillic, gallic, ellagic, hydroquinone); hydroxycinnamic acid derivatives (e.g., p-coumaric, caffeic, ferulic, sinapic); flavonoids (e.g., catechins, proanthocyanins, anthocyanidins and flavons, flavonols and their glycosides); and “tannins” (e.g., plant polymeric phenolics with the ability to precipitate protein from aqueous solutions).

These groups of phenolic compounds share the ability to inhibit microorganisms; therefore, they may have a common mode of action. Although the main subject of this chapter is natural plant-derived antimicrobials, discussion will include findings using synthetic phenolic antioxidants. Phenolics exert antimicrobial activity by injuring lipid-containing membranes, which results in leakage of cellular contents. Antimicrobial activity of phenolic compounds (including synthetic antioxidants) has been proved against several bacteria, recognizing that Gram-positive are generally more sensitive to these compounds (Table 14.3). Branen et al. (1980) reported the inhibitory effect of phenolic antioxidants against several bacteria, including *Salmonella* Senftenberg, *S. Typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Pseudomonas fluorescens*, and *P. fragi*. Kabara (1991) reported minimal inhibitory concentrations (MIC) of BHA in the range of 125 to 250 ppm for *Streptococcus mutans*, *S. agalactiae*, and *Staphylococcus aureus* in laboratory broth with pH 7.0. Antimicrobial activity appeared to be strongly dependent on, among others, microbial species (strain and concentration), type and concentration of phenolic compounds, combination with other antimicrobials, temperature, food additives, and food components (Raccach, 1984). Concentration of phenolic antioxidants with antimicrobial activity in food products was in the range of 30 to 10,000 ppm, yet these compounds are permitted as antioxidants in concentrations generally up to 200 ppm, based on the fat or oil content of the food product. To comply with U.S. federal regulations, Kabara (1991) suggested the use of phenolic antioxidants in combination with other antimicrobials.

It has been reported that some of these phenolic compounds have a wide antimicrobial spectrum (Table 14.3), such as thymol extracted from thyme and oregano, cinnamic aldehyde extracted from cinnamon, and eugenol extracted from cloves (Wilkins and Board, 1989; Beuchat and Golden, 1989; Davidson, 1993). Vanillin, a phenolic compound present in vanilla pods (Figure 14.1), also has antifungal activity (Beuchat and Golden, 1989; Cerrutti and Alzamora, 1996; López-Malo et al., 1995, 1997, 1998). Another phenolic compound present in plants with antimicrobial activity is oleuropein, obtained from green olive extracts (Nychas, 1995; Beuchat and Golden, 1989; Paster et al., 1988; Gourama and Bullerman, 1987; Tassou and Nychas, 1994). Antimicrobial activity of naturally occurring phenols and phenolic compounds from olives, tea, and coffee has been also examined. Nychas (1995) reported that phenolic extracts of black and green tea and/or coffee could be bactericidal or bacteriostatic against *Campylobacter jejuni*, *C. coli*, *Streptococcus mutans*, *Vibrio cholerae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Plesiomonas shigelloides*, *Salmonella* Typhi, *S. Typhimurium*, *S. Enteritidis*, *Shigella flexneri*, and *Shigella dysenteriae*. Moreover, *Pseudomonas fragi*, *Lactobacillus plantarum*, *S. aureus*, *Staphylococcus carnosus*, *Enterococcus faecalis*, and *Bacillus cereus* were inhibited by ethyl acetate extracts from olives and *B. subtilis*, *B. cereus*, and *S. aureus* were inhibited by oleuropein.

Hitokoto et al. (1978) reported that cinnamon bark had the same strong inhibitory effect on the molds *Aspergillus flavus*, *A. parasiticus*, *A. versicolor*, and *A. ochraceus*. Bullerman (1974) also observed this inhibitory effect of cinnamon on an aflatoxin-producing mold, and reported that a

TABLE 14.3
Selected References Testing Antimicrobial Activity of Phenolic Compounds

Microorganism	Antimicrobial	Reference	
Bacteria	Phenolic antioxidants	Rico-Muñoz and Davidson (1983), Shelef and Liang (1982)	
	Mint essential oil	Tassou et al. (1995)	
	Selected spices	Beuchat (1976)	
	Phenolic compounds from spices	Al-Khayat and Blank (1985), Venturini et al. (2002)	
	Oleuropein	Tassou and Nychas (1994)	
	Sage	Shelef et al. (1984)	
	Essential oils from spices	Aureli et al. (1992), Hammer et al. (1999), Baratta et al. (1998), Dorman and Deans (2000)	
	Vanillin	Castañón et al. (1999)	
	Plant essential oils	Deans and Ritchie (1987), Smith-Palmer et al. (1998), del Campo et al. (2000), Elgayyar et al. (2001)	
	Thyme essential oil	Juven et al. (1994)	
	Flavoring agents	Jay and Rivers (1984)	
	Oregano, thyme and essential oils	Paster et al. (1990), Lambert et al. (2001)	
	Yeasts	Phenolic antioxidants	Rico-Muñoz and Davidson (1983)
		Vanillin	Cerrutti and Alzamora (1996), Cerrutti et al. (1997)
Flavoring agents		Jay and Rivers (1984)	
Oregano, thyme, and essential oils		Paster et al. (1990)	
Essential oils		Conner and Beuchat (1984a), Elgayyar et al. (2001)	
Molds	Flavoring agents	Jay and Rivers (1984)	
	Phenolic antioxidants	Fung et al. (1977), Rico-Muñoz and Davidson (1983)	
	Garlic	Graham and Graham (1987)	
	Essential oils from citric	Karapinar (1985)	
	Oregano, thyme, and essential oils	Paster et al. (1990), Paster et al. (1995)	
	Cinnamon, clove, and essential oils	Bullerman et al. (1977), Sebtí and Tantaoui-Elaraki (1994)	
	Thymol and carvacrol	Thompson (1990), Buchanan and Sheperd (1981)	
	Essential oils	Thompson (1990), Mahmoud (1994), Juglal et al. (2002), Elgayyar et al. (2001)	
	Spices and herbs	Llewellyn et al. (1981), Hitokoto et al. (1980), Azzous and Bullerman (1982)	
	Vanillin	López-Malo et al. (1995, 1997, 1998), Cerrutti et al. (1997)	

1% to 2% level of ground cinnamon in broth allowed some growth of *A. parasiticus* but eliminated approximately 99% of the production of aflatoxin. In a later study, Bullerman et al. (1977) demonstrated that the essential oil of cinnamon at a concentration of 200 ppm was inhibitory of growth and subsequent toxin production by *A. parasiticus* and that cinnamic aldehyde, the major component of cinnamon oil, was effectively inhibitory at a level of 150 ppm. Bullerman et al. (1977) reported that cinnamic aldehyde and eugenol, the major constituents of essential oils from cinnamon and clove, respectively, were the active antimicrobial compounds of the oils. However, they do not discard other minor constituents that may also have antimicrobial activity (Table 14.2). Furthermore, Paster et al. (1995) reported that carvacrol and thymol had lesser inhibitory effects on mold growth than extracts from oregano and clove, suggesting that the antimicrobial activity may be the result of several compounds from the extracts. These findings suggest that the phenolic extracts from plants may contain not only phenolics but also other compounds that could possess antimicrobial activity.

Oregano, thyme, and savory have been reported to have substantial antimicrobial activity. The terpenes carvacrol, p-cymene, and thymol are the major volatile components of oregano, thyme, and savory and likely account for the antimicrobial activity (Beuchat, 1976; Farag et al., 1989;

Zaika, 1988). The essential oil of oregano contains up to 50% thymol; thyme has 43% thymol and 36% p-cymene, and savory has 30% to 45% carvacrol and 30% p-cymene (Farag et al., 1989; Farrell, 1990). *B. subtilis*, *Salmonella* Enteritidis, *S. aureus*, *Pseudomonas aeruginosa*, *Proteus morgani*, and *Escherichia coli* were inhibited by carvacrol and thymol at dilutions of >1:2000 (Katayama and Nagai, 1960).

Eugenol has been reported by Al-Khayat and Blank (1985) as one of the most effective natural antimicrobials from plant origin acting as a sporostatic agent: significant reductions of viable *Bacillus subtilis* spores were obtained when exposed to 0.1% to 1.0 % eugenol for 8 days at 37°C. Gingeron, zingerone, and capsaicin have been also found to be sporostatic for *B. subtilis* (Al-Khayat and Blank, 1985). Buchanan and Shepherd (1981) found that 100 ppm thymol inhibited *Aspergillus parasiticus* growth for 7 days at 28°C. Mahmoud (1994) reported that cinnamic aldehyde in lower concentrations than the MIC delayed *Aspergillus flavus* growth for 8 days at 28°C. When studying the relationship between structure and inhibitory actions, Katayama and Nagai (1960) assayed 32 pure phenol compounds. They found that 0.05% eugenol, carvacrol, isoborneol, thymol, vanillin, or salicyldehyde in agar were inhibitory against *B. subtilis*, *S. Enteritidis*, *P. aeruginosa*, *P. morgani*, and *E. coli*, concluding that the presence of an hydroxyl group enhanced the antimicrobial activity. López-Malo et al. (1995) demonstrated that vanillin concentration and the type of agar significantly ($p < 0.05$) affected the radial growth rate of *Aspergillus*. Also, differences among mold response were reported; the most resistant mold to the conditions assayed was *A. niger*, followed by *A. parasiticus*, *A. flavus*, and *A. ochraceus*. The presence of 1000 ppm of vanillin inhibited *A. ochraceus* growth for more than 2 months at 25°C in potato dextrose agar (PDA), whereas growth of *A. niger*, *A. flavus*, and *A. parasiticus* was inhibited by 1500 ppm. Results obtained *in vitro* were corroborated for *A. flavus* and *A. parasiticus* in apple-, banana-, mango-, papaya-, and pineapple-based agars and for *A. ochraceus* and *A. niger* in papaya-, pineapple-, and apple-containing agars. However, inhibitory concentrations of vanillin for *A. niger* and *A. ochraceus* in mango- and banana-based agars were greater than those found in PDA.

Other spices and oils have also been shown to have some antimicrobial activity. Marth (1966) reviewed studies that reported antimicrobial activity by spice oils and extracts of sweet marjoram, laurel, pimienta (chili), coriander, anise, carvone, peppermint, caraway, cardamom, cumin, fennel, celery, dill, and mustard. Many of these preparations seem to be active against only one microbial strain, and some are even active in one study but not in another. The normal inhibitory concentrations used in these studies are very high, indicating little activity. Other species, including rosemary, sage, and turmeric, also possess antimicrobial activities.

Information about antimicrobial effects of essential oils is more abundant. As we said before, although essential oils contain a variety of compounds of different chemical classes, natural phenolics have been reported to be the main antimicrobial compounds. Conner and Beuchat (1984a) found, studying essential oils of plants and spices against several food spoilage yeasts, that each studied yeast responded in a different way, indicating that extracts can present various modes of action or there are diverse yeast metabolism responses to the antimetabolic effect of essential oils. *Rhodotorula rubra* was highly sensitive to cinnamon, clove, garlic, onion, oregano, and thyme oils in concentrations between 25 and 200 ppm; however, *Kloeckera apiculata* was moderately sensitive, and *Torulopsis glabrata* was resistant to most of the oils excluding those of garlic and onion. Paster et al. (1990), evaluating the antimycotic capacity of essential oil of oregano and clove against three strains of *Aspergillus*, indicated that the studied molds differed in their sensibility to the extracts and found that *A. flavus* was sensitive to the essential oil of oregano. Zaika et al. (1983) found that little if any inhibition of growth and acid production by *Lactobacillus plantarum* and *Pediococcus acidilactici* was noted in the presence of 40 ppm oregano oil, whereas levels >200 ppm were bactericidal to both organisms. Also, they reported that bacteria exposed to sublethal concentrations of oregano oil were able to overcome the inhibition and to develop resistance to the toxic effect of oregano oil or oregano.

Essential oils of thyme and oregano were inhibitory to *Vibrio parahaemolyticus*, 25 genera of bacteria, and *S. aureus*; essential oil of sage was inhibitory to *V. parahaemolyticus*, *Bacillus cereus*, *S. aureus*, and *S. Typhimurium*, and rosemary spice extract at 0.1% substantially inhibited growth of *S. Typhimurium* and *S. aureus* (Sofos et al., 1998). Resnik et al. (1996) analyzed the effect of the concentration of vanillin on the growth rate and aflatoxin accumulation of *A. parasiticus*. The growth rate decreased abruptly in the presence of 250 ppm of vanillin, whereas 1500 ppm of vanillin inhibited mold growth during at least 37 days of storage at 28°C. However, 500 ppm of vanillin enhanced the aflatoxin B₁ (AFB₁) and aflatoxin G₁ (AFG₁) accumulation, the toxin levels exceeding those of the control.

OTHER PLANTS AS SOURCES OF ANTIMICROBIAL AGENTS

Among the plants widely consumed in the human diet, garlic, onion, and leek have been recognized and studied for their antimicrobial properties (Beuchat and Golden, 1989). Plants of the *Allium* species, namely garlic and onion, have been extensively revised. Garlic (*Allium sativum*) has been used medicinally for centuries. Many foodborne pathogenic bacteria are sensitive to onion and garlic extracts. *S. aureus*, *B. cereus*, *Clostridium botulinum*, *S. Typhimurium*, and *E. coli* have been adversely affected by garlic extracts (Beuchat and Golden, 1989). The antimicrobial effects reported for garlic and onion were attributed to the allicin concentrations and other sulfur compounds present in their essential oils.

Allicin was described as a colorless oil, extremely pungent, that characterized the principle odor and taste of garlic and onion. It was reported that allicin in concentrations of 1:85,000 in broth was bactericidal to a wide variety of Gram-negative and Gram-positive organisms. Investigations have shown that extracts from *Allium* bulbs inhibit growth and respiration of pathogenic fungi and bacteria. Aqueous extracts from fresh garlic bulbs at levels of 3%, 5%, and 10% inhibited the growth of *B. cereus* on nutrient agar plates by 31.3%, 58.2%, and 100%, respectively (Saleem and Al-Delaimy, 1982). Mantis et al. (1978) studied the effect of garlic extract on *S. aureus* in culture media. They reported that a 5% garlic extract concentration had a germicidal effect on *S. aureus*, whereas concentrations of garlic extract equal to or greater than 2% had a clear inhibitory effect and concentrations less than 1% were not considered inhibitory for this culture. An investigation of the effect of garlic and onion oils on toxin production by *C. botulinum* in meat slurry indicated that these oils, when used in the proportion of 1500 µg/g meat slurry, inhibited toxin production by *C. botulinum* type A. However, the inhibition was incomplete and toxin production by *C. botulinum* type B and type E was not inhibited (DeWit et al., 1979). Yin and Tsao (1999) evaluated the antifungal activity of *Allium* plant (including garlic) extracts against *Aspergillus niger*, *A. flavus*, and *A. fumigatus* and found that garlic was effective as a fungicidal in concentrations between 35 and 104 ppm.

Geraniol, nerol, and citronellol have been also reported as antimicrobial compounds. Mahmoud (1994) found that 1000 ppm inhibited *Aspergillus flavus* growth in nutritive broth (pH 5.5) during 15 days of incubation at 28°C, being the MIC of 500 ppm for the three alcohols. López-Malo and Argáiz (1999) evaluated the effects of pH (6.5, 5.5, 4.5, or 3.5) and citral (3,7-dimethyl-2, 6-octadienal) concentration (0, 500, 1000, 1500, or 2000 ppm) on the growth of *Aspergillus flavus*, *A. parasiticus*, *Penicillium digitatum*, and *P. italicum* in PDA adjusted with sucrose to a_w 0.97. The radial growth rate of molds increased as pH increased and citral concentration decreased. Conversely, reducing pH and increasing citral concentration increased mold germination time. Inhibitory citral concentration depended on pH and differed among molds. *P. digitatum* and *P. italicum* were inhibited with 500 ppm citral at pH 3.5 and 6.5, whereas 1000 ppm were required to inhibit *A. flavus* and *A. parasiticus*. At pH 4.5 and 5.5 inhibitory concentrations varied from 1000 ppm for *Penicillium* to 2000 ppm for *Aspergillus*.

Phytoalexins, low-molecular-weight compounds produced by higher plants in response to microbial infections or treatment of plant tissues with biotic or abiotic elicitors, are also broad-

spectrum antimicrobial agents that probably act by altering the microbial plasma membranes. Their antimicrobial activity is directed against fungi, but they also have an effect on bacteria (Sofos et al., 1998; Smid and Gorris, 1999). More than 200 compounds have been isolated from more than 20 families of plants, the most important chemical classes being isoflavonoids and, to a lesser extent, proteins (chitinases, thionins, zeamatin, thaumatins, etc.). However, the high concentrations needed to exert antimicrobial action in food matrices explain the few examples of the actual use in foods (Sofos et al., 1998).

MECHANISMS OF ACTION

The preceding reports indicate that some spice extracts (essential oils) have a broad spectrum of biological effects, whereas other extracts may be specific toward certain groups of microorganisms, such as Gram-positive or Gram-negative bacteria, or only bacteria, not yeasts or molds. Some essential oils, plant extracts, and oleoresins influence certain biochemical and/or metabolic functions, such as respiration or production of toxins or acids, indicating that the active components in various oils and oleoresins may have different specificity with regard to target sites on or in microbial cells. In most of the research on spices there has been much speculation on the contribution of the terpene fraction to their antimicrobial activity. Few of the studies, however, have gone so far as to attempt to isolate and identify the antimicrobial fraction, and few are on subjects relating to the mechanism by which spices inhibit microorganisms. It seems reasonable that because many of the components of the essential oils, such as eugenol and thymol, are similar in structure to active phenolic antimicrobials, their modes of action could be assumed to be similar.

The possible modes of action of phenolic compounds have been reported in different reviews (Wilkins and Board, 1989; Beuchat, 1994; Nychas, 1995; Sofos et al., 1998; Davidson, 2001). However, these mechanisms have not been completely elucidated. Prindle and Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent. At low concentration phenols affected enzyme activity, especially of those enzymes associated with energy production, whereas at greater concentrations phenols caused protein denaturation. The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds of altering microbial cell permeability, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in its structure and functionality (Fung et al., 1977). Lis-Balchin and Deans (1997) reported that a strong antimicrobial activity (against *Listeria monocytogenes*) could be correlated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde, and thymol. However, when testing isolate compounds against *L. monocytogenes* this correlation was not that easy, concluding that there is a more complex relationship with the chemical composition of the essential oil (which also includes minor components).

Conner and Beuchat (1984a, b) suggested that antimicrobial activity of essential oils on yeasts could be the result of disturbance in several enzymatic systems involved in energy production and structural components synthesis. Once the phenolic compound crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. Juven et al. (1994) found that increasing thyme essential oil, thymol, or carvacrol concentration was not reflected in a direct relationship with antimicrobial effects. However, they reported that after exceeding a certain concentration (critical concentration), a rapid and drastic reduction in viable cells of *S. Typhimurium* was observed. Phenolic compounds could sensitize cellular membranes, and when sites were saturated a serious damage and a rapid collapse of cytoplasmic membrane integrity could be presented, with the consequent loss of cytoplasmic constituents. Ruiz-Barba et al. (1990), using scanning electron microscopy, showed that cells without treatment were smooth compared with those treated with phenols for 24 hours, which appeared rugged and with irregular surfaces. Kabara and Eklund (1991) mentioned that phenolic compound effects could be at two levels, on cellular wall and membrane integrity and on microbial physiologic

responses. Phenolic compounds could also denature enzymes responsible for spore germination or interfere with amino acids necessary in germination processes (Nychas, 1995). Rico Muñoz et al. (1987), after finding that various phenolic compounds have different effects on the membrane of *S. aureus*, concluded that phenolic compounds probably do not share a common mechanism of action and that there might be various targets associated with their antimicrobial effect.

The major antimicrobial constituent of garlic and onion is allicin, along with several other sulfur-containing compounds. Cavallito and Bailey (1944) were the first to isolate the major antimicrobial component from garlic bulbs by steam distillation of ethanol extracts. Later, Cavallito et al. (1945) identified this agent as allicin or diallylthiosulfonic acid. It was noted that allicin was extremely pungent and characterized the principle odor and taste of garlic and onion. The high antimicrobial seen with garlic and onion extracts is probably the result of a high content of allicin and other sulfides in the essential oils of garlic and onion. Wills (1956) observed that inhibition of sulfhydryl enzymes was associated with the presence of the $-SO-S-$ grouping, not $-SO-$, $-S-S-$, or $-S-$ groups. Barone and Tansey (1977) reported that allicin disrupted microbial cell metabolism primarily by inactivation of $-SH$ proteins by oxidation of thiols to disulfides; competitive inhibition of the activity of sulfhydryl components, such as cysteine and glutathione, by binding with them and/or by noncompetitive inhibition of enzyme functions by oxidation of the binding to $-SH$ groups at allosteric sites. Furthermore, Barone and Tansey (1977) reported that the antimicrobial activity of allicin was somewhat nonselective and hypothesized that allicin interfered with electron flow through the disulfhydryl reductase system and inhibited the reductase function by oxidation of sulfhydryl groups within the cell wall, which would result in an “uncoupling” of cell division from cell metabolism, which in turn could lead to an increase in mycelial form in dimorphic yeasts.

FACTORS AFFECTING ACTIVITY

The antimicrobial activities of extracts from several types of plants and plant parts used as flavoring agents in foods have been recognized for many years. However, not many data have been reported on the effect of extracts in combination with other factors on microbial growth. Its potential as a total or partial substitute for common preservatives to inhibit growth of spoilage and pathogenic microorganisms needs to be evaluated alone and in combination with traditional preservation factors or hurdles (mainly storage temperature, pH, water activity, other antimicrobials, modified atmospheres). These results could be very useful in terms of allowing research workers involved in product development under the concept of hurdle technology to assess quickly the impact of altering any combination of the studied variables.

The effect of pH on the antimicrobial activity of natural phenolic compounds is not clearly understood. However, some authors reported a greater effect as the pH was reduced. Sykes and Hooper (1954) found greater effects of phenolics at acid pH values, and they attributed it to the increased solubility and stability of these compounds at low pH. Juven et al. (1994) reported that phenolic active compounds sensitize the cell membrane of *S. Typhimurium* and *S. aureus*, and, when saturation of the sites of action occurs, there is a gross damage and a sudden collapse of the integrity of the microbial cell. They mentioned that at low pH values, the thymol molecule is mostly undissociated and may bind better to hydrophobic regions of the membrane proteins and dissolve better in the lipid phase of the membrane.

Thompson (1990) reported that 1.0 mM carvacrol inhibited at least 7 days at 27°C the growth of *Aspergillus* when the pH of the medium was 4.0 or 8.0; at pH 6.0 only mycelium production reductions (about 50% to 80%) were observed. Thymol (1.0 mM) addition inhibited studied molds at the three evaluated pH levels. Al-Khayat and Blank (1985) reported that 0.03% eugenol reduced *Bacillus subtilis* spores' ability to germinate while lowering the pH of culture medium from 8.0 to 6.0 and mentioned that eugenol was effective at low pH. Juven et al. (1994) found an increased antimicrobial activity of essential oil of thyme and thymol at pH 5.5 in comparison with the one at pH 6.5 and attributed these effects to changes in polar groups distribution of phenolic constituent

of oils between the cytoplasm membrane and the external medium. At low pH the thymol molecule is largely undissociated and therefore more hydrophobic, can be joined better to hydrophobic regions of membrane proteins, and dissolved more easily in the lipid phase. Kabara (1991) mentioned that undissociated phenolic groups are more active as antimicrobials than dissociated forms, suggesting that phenols can act on a wide pH range (3.5 to 8.0). Tassou et al. (1995) found synergistic effects between pH and mint essential oil in *S. Enteritidis* and *L. monocytogenes* inhibition in foods and laboratory media.

López-Malo et al. (1998) reported second-order polynomial equations for describing the lag time for mold (*Aspergillus ochraceus*, *A. niger*, *A. parasiticus*, and *A. flavus*) growth under the effects of pH and vanillin concentration in laboratory media. Contour plots for fixed lag times were obtained with predictive models. For the four molds assayed, the lag time increased as vanillin concentration increased and pH decreased. The effect of the variables was more important for *A. flavus* and *A. ochraceus*. For *A. niger* and *A. parasiticus* the greatest level of vanillin concentration evaluated (1000 ppm) was not enough to cause an effective increment in lag time even at pH 3.0. Results demonstrated that a combination of vanillin with pH reduction had an additive or synergistic effect on mold growth depending on the *Aspergillus* species. *A. ochraceus* was inhibited at pH 3.0 with 1000 ppm of vanillin. Higher concentrations could inhibit the other molds.

Tassou et al. (1995) found that in a salty food, 1.0% mint essential oil drastically affected *S. Enteritidis* growth and reported that salt acted synergistically with mint essential oil. López-Malo et al. (1997) reported mold (*Aspergillus*) inhibitory concentrations of vanillin at selected pH and incubation temperatures. The inhibitory conditions (no growth after 30 days) depended on the type of mold. The most resistant one, *A. niger*, was inhibited with 1000 ppm vanillin at pH \approx 3.0 and incubation temperature \approx 15°C or with 500 ppm at pH \approx 4.0 and temperature \approx 10°C. For *A. ochraceus*, the most sensible, the inhibitory conditions in systems containing 500 ppm vanillin were pH 3.0 and temperature \approx 25°C or pH 4.0 with temperature \approx 15°C. If the pH was increased to 4.0, the vanillin concentration could be \approx 1000 ppm with temperatures lower than 15°C, or 500 ppm if the incubation temperature was reduced to 10°C.

Fungal inhibition can be achieved by combining spices and traditional antimicrobials, reducing the concentrations needed to achieve the same effect. Azzouz and Bullerman (1982) reported that clove was an efficient antimycotic agent against *Aspergillus flavus*, *A. parasiticus*, and *A. ochraceus* and four strains of *Penicillium*, delaying mold growth by more than 21 days. These authors also found additive and synergic effects combining 0.1% clove with 0.1% to 0.3% potassium sorbate, delaying mold germination time. Sebti and Tantaoui-Elaraki (1994) reported that the combination of sorbic acid (0.75 g/kg) with an aqueous cinnamon extract (20 g/kg) was effective to inhibit growth of 151 mold and yeasts strains isolated from a Moroccan bakery product, while 2000 ppm sorbic acid alone was required to inhibit the microorganisms studied. Matamoros-León et al. (1999) evaluated the individual and combined effects of potassium sorbate and vanillin concentrations on the growth of *Penicillium digitatum*, *P. glabrum*, and *P. italicum* in PDA adjusted to aw 0.98 and pH 3.5. These authors found that 150 ppm potassium sorbate inhibited *P. digitatum*, whereas 700 ppm was needed to inhibit *P. glabrum*. Using vanillin, inhibitory concentration varied from 1100 ppm for *P. digitatum* and *P. italicum* to 1300 ppm for *P. glabrum*. When used in combination, MIC isobolograms showed curves deviated to the left of the additive line. Also, calculated Fractional Inhibitory Concentration (FIC) Index values varied from 0.60 to 0.84. FIC index as well as isobolograms showed synergistic effects on mold inhibition when vanillin and potassium sorbate were applied in combination.

APPLICATION IN FOODS

Unfortunately, many of the published reports about the application of phenolic compounds (antioxidants and constituent of extracts and essential oils) as antimicrobials have been accomplished in model and laboratory systems and there are few studies that have been carried out in real foods

(Board and Gould, 1991). The essential oils of spice and plants, as well as their major components, are more effective in microbiological media than when evaluated in real foods (Shelef et al., 1984). In most cases the inhibitory concentrations found in model systems increase significantly when evaluated with the same microorganisms in actual foods and, consequently, few of the applications of phenolic antioxidants as antimicrobials have been successful (Kabara and Eklund, 1991). This reduction in the effectiveness observed *in vivo* represents an important limitation to the use of essential oils and phenolic antioxidants as antimicrobial agents in foods (Juven et al., 1994). The interactions among phenolic groups and proteins, lipids, and aldehydes could explain at least partially the reduction of the antimicrobial effect of essential oils where the major constituents are phenols.

Juven et al. (1972) showed that oleuropein antimicrobial activity could be reduced by the addition of tryptone and/or yeast extract to the culture medium. Tassou and Nychas (1994) demonstrated that inoculum size, oleuropein concentration, and pH influenced significantly *S. aureus* growth and lag time and proved that the efficiency of phenolic compound antimicrobial action was reduced in foods with relatively low protein content. In studies with antioxidants in milk defatted solids, Cornell et al. (1971) found that BHA was bound to the casein through hydrophobic interactions and should probably decrease its antimicrobial activity. Rico-Muñoz and Davidson (1983), studying casein and corn oil effects in phenolic antioxidant antimicrobial activity, reported that casein did not have effect on *S. cerevisiae* or *S. aureus* growth and slightly reduced growth of *P. fluorescens*. However, casein presence dramatically reduced the antimicrobial activity of BHA. For *P. citrinum* the inhibitory activity of 200 ppm BHA was almost eliminated in the presence of 6% and 9% casein; however, for *A. niger* the addition of 3% casein caused the antimycotic effect of the antioxidant to disappear. The same authors reported that the presence of corn oil or Tween 80 reduced or eliminated the antimicrobial activity of BHA and TBHQ. The principal cause of antimicrobial activity loss could be the solubilization of these compounds in the lipid phase of the medium, reducing its availability to act as antimicrobial. Aureli et al. (1992), evaluating the antimicrobial capacity of thyme against *L. monocytogenes* growth in model systems and in a real food, found that essential oil antilisteric efficiency decreased when used *in vivo* (ground pork meat) in comparison with the behavior in laboratory media (solid and liquid).

Spencer et al. (1988) reported that the interaction or complex between phenols and proteins depends partially on protein characteristics, pH, and the phenolic group containing molecules. This interaction takes place by hydrogen bonds between phenolic groups and peptides and also by hydrophobic interactions. The interactions of aldehydes with proteins have been extensively studied because protein addition to aldehyde solutions can decrease the effective concentration of these groups (Cha and Ho, 1988; Hansen and Heinis, 1991; Montgomery and Day, 1965). Citral (lemon flavor component) concentration was reduced almost 100% when 5% of casein or soy protein isolate was added in aqueous solutions. Of initial vanillin concentration, measured by high-performance liquid chromatography, 68% was lost after 26 hours in drinks containing aspartame (Tateo et al., 1988). Hussein et al. (1984) reported that vanillin concentration in aqueous solutions was reduced 73% after 25 hours at 26°C when aspartame was added. The reduction in vanilla flavor by the reduction of vanillin concentration also had been reported when adding faba-bean proteins, sodium caseinates, or milk whey protein concentrate (Hansen and Heinis, 1991; Ng et al., 1989a, b; Barr, 1990).

The levels of essential oils or plant extracts needed to cause a similar antimicrobial effect in food products in comparison with laboratory media are considerably higher. These greater concentrations of natural antimicrobials may modify the sensory characteristics of the product making it unacceptable. The use of natural antimicrobials in combination with other environmental stress factors not only can enhance their antimicrobial properties but also makes possible the development of products that consumers are demanding, reducing the amounts of synthetic or natural antimicrobials needed to assure microbial stability.

It had been demonstrated that vanillin (4-hydroxy-3-methoxy-benzaldehyde) inhibited microbial growth in laboratory media and fruit purées stored at 25°C to 27°C (López-Malo et al., 2000).

Promising results have been obtained by Cerrutti et al. (1997) and Cerrutti and Alzamora (1996) in strawberry (pH 3.4, a_w 0.95) and apple purées (pH 3.5, $a_w \cong 0.99$) preserved by combined factors. Strawberry purée with 3000 ppm vanillin and reduced a_w inhibited the native and inoculated flora (*Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Schizosaccharomyces pombe*, *Pichia membranefaciens*, *Botrytis* species, *Byssoschlamys fulva*, *Bacillus coagulans*, and *Lactobacillus delbrueckii*) for at least 60 days of storage at 25°C. In apple purée with 2000 ppm vanillin, a germicide effect was observed on inoculated *S. cerevisiae*, *Z. rouxii*, and *D. hansenii*. Cerrutti and Alzamora (1996) also reported that 3000 ppm of vanillin in a banana purée with pH 3.4 and a_w 0.98 were inhibitory for *S. cerevisiae*, *Z. rouxii*, and *D. hansenii*. Castañón et al. (1999) evaluated the effects of vanillin (1000 or 3000 ppm) or potassium sorbate (1000 ppm) addition on the microbial stability during storage at 15°C, 25°C, or 35°C of banana purée preserved by combined methods (a_w 0.97, pH 3.4). Native flora (standard plate, yeasts, and molds) counts during storage at different temperatures in the control purées (without antimicrobial addition) and in those containing 1000 ppm vanillin demonstrated that a reduced storage temperature (15°C) was not enough to detain or delay the spoilage of the purée. After 6 days of storage at 15°C, native yeasts and molds reached counts of 10^4 cfu/g (about three logarithmic cycles more than the initial count) and the purées were at this time sensorily unacceptable (odor and textural changes accompanying the microbial spoilage). The addition of 1000 ppm vanillin increased the lag phase up to $\cong 16$ days at 15°C, and the time to detect the microbial spoilage was extended to around 21 days. In the presence of 3000 ppm of vanillin or 1000 ppm potassium sorbate no microbial growth (<10 cfu/g) was detected after 6 days and up to 60 days of storage (15°C, 25°C, or 35°C). Results obtained by Cerrutti and Alzamora (1996), Cerrutti et al. (1997), and Castañón et al. (1990) demonstrated that addition of vanillin in combination with a slight reduction of a_w and pH may be a promising method for fruit purée preservation and confirmed antimicrobial properties of vanillin. They found that protein and fat contents of the fruit partially determined the vanillin concentration necessary to obtain a sound product. The relatively high protein (1.2%) and fat (0.3%) contents in banana compared with those of other fruits explain the necessity of a higher vanillin concentration to obtain the same antimicrobial effects as in other fruits.

Castañón et al. (1999) reported results of sensory evaluation of banana purées containing 3000 ppm of vanillin or 1000 ppm of potassium sorbate. The mean scores corresponded to products with a good overall acceptability with scores around 6 ("like slightly"). The purées were significant different ($p < 0.05$) in odor (the one with vanillin was preferred) and flavor (the one with potassium sorbate was better), and there was not significant difference ($p < 0.05$) in color and overall acceptability. The flavoring characteristics of vanillin are well accepted and have demonstrated compatibility with many fruits in concentrations up to 3000 ppm (Cerrutti and Alzamora, 1996). The microbial stability of mango juice (pH 4.9) supplemented with extracts of ginger (antimicrobial compounds: zingerone, gingerol, and shogaol) and nutmeg (antimicrobial compounds: myristicin and sabinene) was investigated during 3 months at room temperature by Ejechi et al. (1998). The combination of heating (15 minutes at 55°C) and 4% v/v of each spice inhibited microbial growth (yeasts and nonspore-forming bacteria) and produced a product with acceptable taste. Surface disinfection of tomatoes using cinnamic aldehyde was studied by Smid et al. (1996). Whole tomatoes were dipped 30 minutes in a solution containing 13 mM cinnamic aldehyde and then stored at 18°C in sealed plastic bags. The combination of the treatment with the natural phenolic compound and packaging under modified atmosphere reduced spoilage-associated fungi and bacteria on the surface of the tomatoes, increasing the shelf life up to 11 days.

FINAL REMARKS

Phenolic compounds are naturally present in vegetable products and sometimes in considerable quantities. Few of the natural phenols are toxic, and only some have been reported as a cause of allergic reactions in animals. In the few occasions in which phenolic compounds have been harmful

TABLE 14.4
Factors that Influence Plant-Derived Antimicrobial Selection and Effectiveness

Antimicrobial characteristics

Toxicologic and legal aspects, solubility, sensory impact, cost

Possible synergistic, antagonistic, or additive interaction effects with other antimicrobial factors

Composition of food

Moisture content, fat and protein content, water activity and pH, presence of other inhibitors (acids, salts, smoke, antimicrobials), interactions with food matrix and other food additives

Initial contamination level

Sanitary conditions of ingredients and raw materials, sanitary conditions of equipment, processing conditions, type of potential growing microorganisms

Handling and distribution

Length of storage, temperature of storage, packaging

Source: Adapted from Giese (1994).

to humans, an abnormal consumption of vegetable origin phenols or consumption of abnormal phenols for the diet has been identified as a possible cause (Singleton, 1981). Natural phenolic compounds can be classified as common, uncommon, and rare, depending on their occurrence in vegetables. Phenols that are highly toxic for animals fall within the third category, whereas those that are found in considerable quantities in nature (“common” phenols) have very low toxicity (Singleton, 1981). It has been reported that phenolic compound consumption can affect nutritional aspects in human and animals. However, it has been found that many phenols exhibit beneficial effects in some disorders such as cancer (Nychas, 1995). It is recognized that consumption of fruit and vegetables and other sources of phenolic compounds, such as tea, reduces propagation of various forms of human cancer and other diseases such as cirrhosis, emphysema, and arteriosclerosis (Weisburger, 1992; Newmark, 1992).

Inhibition, or lack thereof, is obviously a function of the test conditions and the microorganism tested, which means that studies with the same spice may yield different results. Zaika (1988) and Davidson and Parish (1989) published review papers that included a review of methods for determining the antimicrobial activity of spices and herbs. These reviews should serve to standardize future testing and reporting of results. Plant-derived antimicrobials are not yet fully exploited. The use of spices, herbs, plants, essential oils, and related phenolic compounds is limited as a result of the high MICs in actual foods with high protein and/or fat contents, which impart undesirable flavor and/or odor. These undesirable effects can be minimized if the natural compound is used in combination with other environmental stress factors in the frame of the “hurdle technology.” Table 14.4 presents some general guidelines to be followed for selecting plant-derived antimicrobials (Giese, 1994). In this way, considering the consumers’ interest in more “natural” foods, the potential for applications in minimally processed fruit and vegetables appears to be good (Alzamora et al., 1995). However, for a wider and more rational use of these natural compounds, some points should be addressed:

- The extraction methodology
- The response of key microorganisms to the multitarget preservation system *in vitro*, and then the evaluation of the efficacy *in vivo*, that is, in the food product (Gould and Jones, 1989).
- The inhibitory activities of naturally derived antimicrobials used at low concentration (compatible with the food flavor), alone or in combination with other hurdles (Gould and Russell, 1991).

- The incorporation of the active principles through the addition of the plant, the herb, or the spice, or, on the contrary, the corresponding “extract” in preference to the highly purified natural antimicrobial compound (Sofos et al., 1998; Wagner and Moberg, 1989).
- The form in which the plant, herb, spice, essential oil, or components will be incorporated into the foods without adversely affecting sensory, nutritional, and safety characteristics and without increasing significantly the formulation, processing, or marketing costs of the minimally processed product to which they are added (Beuchat and Golden, 1989).

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15 Naturally Occurring Compounds – Animal Sources

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CONTENTS

Introduction	455
Lactoperoxidase	456
Molecular Properties	456
Occurrence and Biosynthesis	456
Isolation and Purification	456
Chemistry and Structure	457
Stability	457
Antimicrobial Activity	458
Mode of Action	458
Specificity	458
Applications in Food	459
Transferrins	460
Lactoferrin, Lactoferricin B, and Activated Lactoferrin	460
Introduction	460
Molecular Properties	461
Occurrence and Biosynthesis	461
Isolation and Purification	462
Physicochemistry	462
Structure	462
Antimicrobial Activity	463
Mode of Action	463
Specificity	466
Applications in Foods	468
Safety and Tolerance	470
Ovotransferrin	471
Immunoglobulins	472
Lactoglobulins	473
Introduction	473
Molecular Properties	473
Occurrence	473
Structure	473
Biosynthesis	474
Isolation and Purification	474
Stability	475
Antimicrobial Activity	475

Mode of Action	475
Specificity	476
Applications in Food	476
Ovoglobulins	476
Introduction	476
Molecular Properties	477
Occurrence and Biosynthesis	477
Isolation and Purification	477
Chemistry and Structure.....	477
Stability.....	478
Antimicrobial Activity	478
Mode of Action	478
Specificity	478
Applications in Food	479
Other Antimicrobials of Animal Origin	479
Avidin.....	479
Introduction	479
Molecular Properties	479
Occurrence and Biosynthesis	479
Isolation and Purification	480
Chemistry and Structure.....	480
Stability.....	480
Antimicrobial Activity	480
Mode of Action	480
Specificity	481
Applications in Food	481
Lactolipids.....	481
Introduction	481
Molecular Properties	482
Occurrence and Biosynthesis	482
Isolation and Purification	482
Chemistry and Structure.....	482
Stability.....	483
Antimicrobial Activity	483
Mode of Action	483
Specificity	483
Applications in Food	484
Defensins.....	484
Introduction	484
Molecular Properties	484
Occurrence and Biosynthesis	484
Chemistry and Structure.....	485
Antimicrobial Activity	485
Mode of Action and Specificity	485
Applications in Food	485
References	485

INTRODUCTION

Natural antimicrobials include agents found in plants, microbes, insects, and animals (Naidu, 2000; Sofos et al., 1998). This chapter focuses on the natural antimicrobials that exist in animals where they evolved as host defense mechanisms and may exhibit antimicrobial activity in foods as natural ingredients or may be extracted and used as additives in other foods. The discussion in this chapter targets mainly those antimicrobials isolated from milk, especially of bovine origin, and from poultry eggs because these are the major food products produced by animals for human consumption that possess antimicrobial activity devised by nature for protection and immune development in the neonate. The antimicrobials isolated from these products are generally broad-spectrum agents providing protection to the neonate against bacteria, fungi, parasites, and viruses; in some cases they are the only means of transferring protective factors to the unborn or newly born young (i.e., bovine colostrum is the only source of immune factors including antibodies from mother to newborn) (Floris et al., 2003). Maternal transfer of immune factors and antimicrobial substances provides the offspring with acquired immunity, leaving them relatively immunocompetent against diseases. Substances with antimicrobial properties from the mother, such as lactolipids in bovine milk, are not strictly immune factors and may possess both nutritional and protective qualities.

Antimicrobial substances present in milk, milk products, and eggs include lactoferrin/lactoferricin B/activated lactoferrin, lysozyme, lactoperoxidase, and lactoglobulins in bovine milk, and ovotransferrin, lysozyme, ovoglobulin, immunoglobulin Y (IgY), and avidin in poultry eggs. All of these compounds are polypeptides. The proteinaceous nature of these molecules partially explains the selective targeting of microbial membranes common to the mode of action displayed by these compounds. Damage to the microbial membrane may be through many mechanisms, some of which include the generation of oxidation products, blocking of receptor-ligand interactions, iron deprivation, and antibody-mediated mechanisms (complement activation, agglutination, opsonization, adherence-blocking, or neutralization).

In general, animal-derived antimicrobials have been known for many years, and thus there has been much interest in the isolation and purification of these compounds to be used as food adjuvants. Major challenges faced by the industry include difficulty in the production of these compounds in large enough quantities to be effective in a food and the incorporation of these compounds in foods while minimizing undesirable interactions, undesirable effects, or subsequent inactivation of their desirable effect. It should also be noted that the use of these compounds as adjuvants to foods may not have the same effectiveness as if the compound were a natural ingredient in food. This is especially true in milk, where the total antimicrobial activity is not necessarily a function of individual antimicrobials but probably the additive or synergistic effects of multiple antimicrobials (i.e., lactoferrin, lactoperoxidase, lactoglobulins, etc.). Despite the challenge of obtaining effective activity of antimicrobial compounds in a food matrix, there has been commercial application of various compounds isolated from animal products including fortification of dairy products with milk antibodies, fortification of dairy products including milk with substrates to facilitate lactoperoxidase production, and use of monoacylglycerols from milk as biopreservatives in many foods. Furthermore, there are many proposed applications of antimicrobial compounds in foods or consumables including the following: (1) passive immunization with milk- and egg-derived antibodies; (2) addition of transferrins, lactoperoxidase, and immunoglobulins in oral health-care products; and (3) use of lactolipids, immunoglobulins, and transferrins as ingredients in infant formulas. Products derived from animal sources, and in particular eggs and milk, are generally recognized as safe (GRAS) compounds; however, these products do need to be evaluated for potential allergiologic properties in sensitive individuals. The following sections provide a summary of the most important naturally occurring antimicrobial compounds found in animal tissues and in particular bovine milk and poultry eggs.

LACTOPEROXIDASE

Lactoperoxidase (LP), a hemoprotein present in milk, tears, and saliva (Tenovuo and Pruitt, 1984), is the most abundant enzyme in bovine milk (Reiter, 1985a). The peroxidase activity associated with bovine milk was first demonstrated by Arnold in 1881, and the protein termed lactoperoxidase was isolated by Theorell and Akeson in 1943 (Naidu, 2000). The lactoperoxidase–thiocyanate–hydrogen peroxide interaction constitutes what is referred to as the LP system, wherein hydrogen peroxide serves as a substrate for LP in oxidizing thiocyanate (SCN^-) and iodide ions, resulting in the generation of highly reactive oxidizing agents (Thomas, 1985; Naidu, 2003). The association of LP with the inhibition of microbial growth was first demonstrated by Wright and Trammer (1958), whereas characterization of the complete LP system including enzymes and substrates occurred later (Jago and Morrison, 1962; Reiter et al., 1963). Additionally, the LP system possesses hexokinase and glyceraldehyde-3-phosphate dehydrogenase activities, which may contribute to the antimicrobial action of the system (Carlsson et al., 1983). The LP system has the ability to inhibit bacteria, fungi, parasites, and viruses and thus is considered a broad-spectrum natural antimicrobial contributing to protecting the gut of weaning calves from enteric pathogens, protecting the mammary gland from disease, and indeed preserving milk (Bjorck et al., 1979; Reiter and Bramley, 1975; Reiter et al., 1980).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

LP is synthesized and secreted by ductal epithelial cells of the mammary gland and other exocrine glands (Parkos, 1997). The compound constitutes approximately 1% (10 to 30 $\mu\text{g}/\text{ml}$) of the whey proteins in the milk (Reiter, 1985a,b) and levels may be influenced by feeding practices, udder irritation, and estrogen levels (Janota-Bassalik et al., 1977; Kern et al., 1963; Kiermeier and Kuhlmann, 1972). The level of LP in bovine milk is about 20 times higher than that of human milk and changes constantly during the postpartum period. Thiocyanate, which is required for the antimicrobial activity of the LP system, may be present in significant amounts in saliva, milk, and airway secretions, whereas hydrogen peroxide may be generated by microbial flora, usually bacteria, resident in sites such as the oral cavity, respiratory tract, or mammary gland or *in situ* by epithelial cells in these regions (Reiter and Perraudin, 1991). In bovine milk, the initial concentration of LP in colostrum is low, increasing to a peak at 4 to 5 days postpartum, after which it declines to a level considered relatively high and remains unchanged at that level during lactation (Kiermeier and Kuhlmann, 1972). To combat infections, the concentrations of LP and SCN^- increase in milk from infected bovine udders as compared with normal, healthy udders (Janota-Bassalik et al., 1977).

Isolation and Purification

The essential steps involved with isolation of LP include casein precipitation with rennet, adsorption of whey proteins via ion-exchange methods, elution, fractionation, and final purification (Naidu, 2000). Early methods of LP isolation, as described by Paul et al. (1980), involved the coagulation of milk with rennin, mixing of resulting whey with the ion-exchanger, washing, and ion-exchange through a column of sodium acetate buffer. Purification of LP isolated this way was achieved by further elution through columns with sodium acetate, concentration of the eluate, fractionation by chromatography, and finally subfractionation using borate. A modification of this method involved concentrating the eluate using ultrafiltration, salting out with ammonium sulfate, and doing a final purification with isoelectric focusing in a borate-polyol system (Denisova et al., 1986). Subsequent research improved purification methods with preparative chromatography, analytical electrophoresis, and image processing (Ferrari et al., 1995). Hahn et al. (1998) developed a fractionation scheme based on the binding capacity of LP-adsorped, cation-exchangers to the immunoglobulin,

IgG. This research suggested that cation-exchangers were successful for large-scale purification of bovine whey proteins and that binding capacity was the highest when pure bovine IgG was used. More recently, Ye et al. (2000) discovered a rapid method for isolation of LP with use of a strong anion exchanger, quarternaryaminoethyl-Toyopearl, which facilitates adsorption of proteins from whey without processing steps such as dialysis with water or a weak buffer.

Chemistry and Structure

It has been determined that bovine LP is comprised of a single peptide chain, with eight disulfide bonds contributing to the rigidity of the molecule (Sievers, 1980). The single polypeptide chain contains 612 amino acid residues with a molecular weight of about 80 kDa (Cals et al., 1991; Paul and Ohlson, 1985). The polypeptide chain contains 15 half-cystine residues and 4 or 5 potential *N*-glycosylation sites, and carbohydrate moieties account for almost 10% of the molecular weight (Cals et al., 1991). LP is a heme-containing enzyme that shares 50% to 70% amino acid sequence homology (particularly among the active-site related residues) with myeloperoxidase, thyroperoxidase, and eosinophil peroxidases (Dull et al., 1990), all of which have a protoporphyrin IX catalytic center (Fenna et al., 1995; Sievers, 1979). The covalent linkages between heme and the protein are well represented in the myeloperoxidase X-ray crystal structure (Fenna et al., 1995; Zeng and Fenna, 1992); however, little work has been done concerning the crystallographic data for LP. Although the details regarding the heme-binding site in LP have not been established, the heme structure has been studied in terms of its electron transfer mechanisms (Nakamura et al., 1986) because the heme moiety is essential for the development of the oxidation–reduction reaction associated with LP activity. The presence of an odd number of half-cystines supports the theory that a heme thiol is released from this enzyme by a reducing agent and suggests that the heme is bound via disulfide links to the peptide chain (Naidu, 2000), indicating that there are no free thiol groups present in the enzyme molecule (Ekstrand, 1994). The iron content of LP is 0.07%, which corresponds to 1 iron atom per LP molecule as part of the heme group (Kussendrager and van Hooijdonk, 2000). The molecular conformation of LP is thought to be stabilized by the strong binding of a calcium ion (Kussendrager and van Hooijdonk, 2000). Although earlier research (Sievers, 1980) revealed that leucin is found on the N-terminus of the LP polypeptide chain, Watanabe et al. (2000) found that different preparations of natural LP may have different N-terminal amino acid residues. This heterogeneity may be a result of variation in terms of isolation methods (i.e., disc-electrophoresis and ion-exchange chromatography); however, the finding suggests that the change in N-terminus structure does not affect the conformation of LP. Other findings of this research using circular dichroism (CD) revealed that the secondary structure (α -helix) of LP is influenced by peptide bonds and that the protein is rich in β -sheet structure (Watanabe et al., 2000).

Stability

It was reported that the LP system stored in airtight containers lost only 35% of the initial thiocyanate concentration during 18 months and that the system was strong enough to kill 10^6 CFU/ml of four test organisms. When the LP system was stored in the presence of air it lost thiocyanate activity after 7 days, but after 516 days it was still able to kill inocula of 10^6 CFU/ml *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* and *Escherichia coli* within 2 hours, 4 hours, and 1 week, respectively (Bosch et al., 2000). It has been shown that during pasteurization, whole milk loses about 75% of its LP activity, whereas the purified LP was rendered unstable after 15 minutes of exposure (Wutrich et al., 1964). Research (Herandez et al., 1990) indicated that heat denaturation of LP in milk, whey, permeate, and buffer started at about 70°C (close to the temperature at which the native structure of the enzyme unfolds to follow first-order kinetics) and that the calcium ion concentration influenced the heat sensitivity of LP. The heat stability of LP is lower under acidic (pH 5.3) conditions and may be related to the release of calcium from the molecule

(de Wit and van Hooijdonk, 1996). LP is deactivated during storage at pH 3 with partial denaturation at $< \text{pH } 4$, whereas there is no deactivation of the enzyme at values of up to pH 10 (de Wit and van Hooijdonk, 1996; Paul and Ohlsson, 1985; Hernandez et al., 1990; Watanabe et al., 2000). The optimum pH for the LP catalyzed reaction lies between 5 and 6, and, based on this, it was concluded that determination of LP should be conducted in these ranges (Bardsley, 1985). LP has been found to be quite resistant to proteolytic enzymes including trypsin and thermolysin, although chymotrypsin was found to inactivate LP relatively slowly (Paul and Ohlsson, 1985; Sievers, 1979). Gothefors and Marklund (1975) found that LP is not inactivated by the gastric juice of an infant (pH 5) but that pepsin at pH 2.5 inactivated LP. The compound appears to be sensitive to light when in the presence of riboflavin (Hernandez et al., 1990), whereas adsorption of LP to certain surfaces may cause a significant decrease in activity (Paul and Ohlsson, 1985).

ANTIMICROBIAL ACTIVITY

Mode of Action

LP is an enzyme with a primary function to oxidize thiocyanate and some halides (I^- and Br^-) at the expense of H_2O_2 to generate products that kill or inhibit the growth of many species of microorganisms (Bosch et al., 2000; Kussendrager and van Hooijdonk, 2000). The reaction mechanism involved in the generation of oxidative products is systematic. The first step is the initiation of the resting LP (Fe^{3+}) to its ground state by H_2O_2 followed by propagation reactions including the conversion of LP from its ground state to the compound I state (the compound that oxidizes SCN^- and halides) (de Wit and van Hooijdonk, 1996). At low SCN^- and halide concentrations, compound I reacts with any one-electron donors that are present to form compound II, which is continually reduced to the ground state. With an excess of H_2O_2 , compound II may react to form compound III. This leads to a ferrylperoxidase adduct and irreversible inactivation of LP. The oxidation of SCN^- is catalyzed by LP and leads to temporary oxidation products, which are responsible for antimicrobial activity (de Wit and van Hooijdonk, 1996). With the oxidation of SCN^- , the generation of OSCN^- (hypothiocyanate) and HOSCN (hypothiocyanous acid) are in equilibrium, and at the pH of maximal LP activity (pH 5.3), they exist in equal quantities. Both oxidized forms are antimicrobial, although there is evidence that hypothiocyanous acid is of stronger activity (Thomas, 1985). The oxidation of sulfhydryl (SH) groups of microbial proteins by OSCN^- (hypothiocyanate) and HOSCN (hypothiocyanous acid) is considered to be the key to the antimicrobial action of the LP system (Aune and Thomas, 1978); however, these forms do not appear to oxidize the SH groups of milk proteins (de Wit and van Hooijdonk, 1996). The structural damage to microbial cytoplasmic membranes through oxidation of SH groups causes leakage of potassium ions, amino acids, and peptides into the medium as well as inhibition of the uptake of glucose, amino acids, purines, and pyrimidines and subsequent synthesis of proteins, RNA (ribonucleic acid), and DNA (deoxyribonucleic acid) (Reiter and Perraudin, 1991).

Specificity

Mammalian cells are not affected by antimicrobial products of the LP system; in fact it is suggested that LP protects human cells against the toxic effects of H_2O_2 (Reiter and Perraudin, 1991). Although there are contrasting views, it appears that LP adsorption to the microbial cell surface is necessary to elicit antimicrobial effects (Tenovuo and Knuutila, 1977). The cytoplasmic membrane and/or the cytoplasm are considered to be the major targets for antimicrobial products generated by the LP system (Naidu, 2000). Accessibility of products generated by the LP system to the interior of the microbial cell may be limited by the cell wall and membrane, although they are not entirely excluded (Naidu, 2000). The cytoplasmic membranes of bacteria and cell walls of Gram-positive bacteria may be altered by antimicrobial products from the LP system (Marshall and Reiter, 1980). Some of the alterations that may occur at the biological barriers protecting bacteria include damage

to the inner membrane resulting in extensive leakage of amino acids, hydrogen ions, and other molecules essential for bacterial survival (Law and John, 1981); inhibition of amino acid transport; and inhibition of glucose and oxygen transport and uptake (Hamon and Klebanoff, 1973; Slowey et al., 1968). Another antimicrobial effect from the products of the LP system is inhibition of several glycolytic enzymes (e.g., hexokinase, aldolase, pyruvate kinase, etc.). The antimicrobial products of the LP system have a varying effect on different groups of microorganisms. The products from the LP system could exhibit static and/or cidal activity on bacteria, fungi, viruses, and parasites, and the molecular mechanism(s) of inhibition (oxidative killing, blockage of glycolytic pathways, or cytopathic effects) would be dependent on the type of organism, type of electron donor present, temperature, pH, and other factors (Naidu, 2000). Gram-negative, catalase-positive bacteria (pseudomonads, coliforms, salmonellae, shigellae, etc.) are not only inhibited but killed depending on pH, temperature, incubation time, cell density, etc. of the substrate (Reiter and Perraudin, 1991; de Wit and van Hooijdonk, 1996). Gram-positive, catalase-negative bacteria (streptococci, lactobacilli, etc.) are generally inhibited but not necessarily killed by the LP system, and the difference in their sensitivity may be explained by the differences in biological barriers (Reiter and Perraudin, 1991; de Wit and van Hooijdonk, 1996). Products of the LP system appear to cause more extensive damage to the inner membrane of Gram-negative than do Gram-positive bacteria (Marshall and Reiter, 1980). Gram-negative bacteria appear to be more sensitive to inactivation by hypothiocyanate than are Gram-positive cells, especially at low temperatures, and are also more sensitive to pH-dependent killing (Naidu, 2000; Pruitt and Reiter, 1985). The antifungal effects of the LP system have been demonstrated against *Candida tropicalis*, *C. albicans*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Rhodotorula rubra*, *Mucor rouxii*, *Byssoschlamys fulva*, and species of *Geotrichum* (Hamon and Klebanoff, 1973; Lehrer, 1969; Popper and Knorr, 1997). Additionally, Doyle and Marth (1978) observed the ability of the LP system to degrade aflatoxin in the presence of salt. Antiviral properties of the LP system have also been displayed against RNA (poliovirus) and DNA (vaccinia) viruses (Belding et al., 1970). Courtois et al. (1990) found the LP system to be effective against HSV1 (herpes simplex virus 1) and the activity to be time-dependent. Inhibition of human immunodeficiency virus has also been demonstrated (Pourtois et al., 1990; Yamaguchi et al., 1993).

APPLICATIONS IN FOOD

The utilization of the LP system as a natural biopreservative in food, in particular dairy products, has increased significantly since the implementation of industrial processes for LP isolation from milk and whey (Kussendrager and van Hooijdonk, 2000). Milk may be cooled and stored for long periods prior to distribution and as such is susceptible to psychrotrophic spoilage, especially by pseudomonads surviving pasteurization (Naidu, 2000). Another and more severe problem originates in developing countries where milk may be unpasteurized and even uncooled prior to distribution and as such may spoil faster and even allow pathogenic organisms to proliferate. Introducing substrates for the production of antimicrobials from the LP system would be a method by which the milk may be protected from contaminating organisms. A two-enzyme coupled system of β -galactosidase and glucose oxidase was developed by Bjorck (1976) to activate the LP system and improve the storage of raw milk. Activation of the LP system by addition of hypothiocyanate and H_2O_2 resulted in considerable reduction of the bacterial flora in the milk and prevented the growth of psychrotrophic bacteria for up to 5 days without altering the physicochemical properties of the milk (Bjorck, 1978). An alternative to preserving milk with LP may be achieved by combining the activation of the LP system with acidification and subsequent souring of the milk (Kangumba et al., 1997; Wray and McLaren, 1987). This method was also found effective against pathogenic microorganisms, especially *Listeria monocytogenes* (Kamau et al., 1990; Kangumba et al., 1997; Rodriguez et al., 1997). Boussouel et al. (2000) concluded that the addition of LP in combination with nisin as a stepwise addition after the maximum inhibitory effect of nisin was reached inhibited *L. monocytogenes* in milk. The addition of LP to starter cultures for yogurt production has also

been studied (Nakada et al., 1996), and results indicated that the acid production was inhibited but the culture was not killed; as such, a new type of yogurt was produced with extended shelf life. A review of potential and actual application of LP systems in dairy products by de Wit and van Hooijdonk (1996) indicated that raw milk could be preserved for 4 days at 4°C and the shelf life could be extended for 3 days at 10°C; the shelf life of pasteurized milk could be extended to 21 days at 10°C; the shelf life of cheese milk could be extended to 8 days at 4°C to 7°C; and, the acidity of yogurt could be suppressed for 14 days at 20°C.

Another major application of LP systems has been described for oral care products (Pellico and Montgomery, 1989); indeed toothpaste (Biotene™) containing complete LP systems (LP/SCN⁻/H₂O₂) has been on the market for a number of years (Kussendrager and van Hooijdonk, 2000). Johansen et al. (1997) evaluated the effects of LP on model biofilms of several oral bacteria and found that the LP combined with oxidoreductases was bactericidal to the biofilms, and when combined with polysaccharide-hydrolyzing enzymes the mixture was able to not only kill but remove the biofilms. Work done with ground beef revealed that LP systems controlled pathogenic contamination and as such have the potential for incorporation into ground beef products as a “natural preservative” (Kennedy et al., 2000). McLay et al. (2002) confirmed that LP systems may be considered as natural antimicrobials for incorporation into food systems including ground beef.

TRANSFERRINS

LACTOFERRIN, LACTOFERRICIN B, AND ACTIVATED LACTOFERRIN

Introduction

Lactoferrin (LF), also termed lactotransferrin or lactosiderophilin (Nagasawa et al., 1972), is an iron-binding, bioactive glycoprotein of the transferrin family that contributes to the control of iron in biological fluids. The transferrin family is subdivided into two branches: the melanotransferrins and the soluble glycoproteins that apart from lactoferrin also include two avian transferrins: the serum transferrin and the ovotransferrin (Baker and Lindley, 1992; Thakurta et al., 2003). The functional properties, including antimicrobial activity, of LF are mainly associated with its ability to bind two Fe³⁺ in combination with two CO₃²⁻ ions. It is found mainly in milk, in the mucosal surfaces (e.g., intestinal epithelial cells) and exocrine secretions of mammals such as saliva, tears, and seminal fluid, and in the secondary granules (vesicles) of polymorphonuclear neutrophils or lymphocytes (Levy, 1996; Levay and Viljoen, 1995; Lonerdal and Lyster, 1995; Naidu, 2000; Van der Strate et al., 2001). Human and porcine milk contain significantly higher concentrations of LF (almost 10 times) than does mature bovine milk, (Masson and Herremans, 1971; Playford et al., 2000; Reiter, 1978a; Sanchez et al., 1988); however, the highest levels of LF exist in bovine colostrum, whereas increases may also be observed in mature milk after mastitic infection (Reiter and Oram, 1967; Mandel and Ellison, 1985; Hagiwara et al., 2003; Playford et al., 2000). LF has an important role in many physiologic pathways, such as adsorption of metal ions in the intestinal tract (Davidson and Lönnerdal, 1986; Davidson et al., 1990, 1994; Naidu, 2000), assistance in digestion and utilization of micronutrients and macronutrients from the milk (Lönnerdal, 2003), suppression of myelopoiesis (Broxmeyer et al., 1987), protection of the intestinal flora in young animals against enteropathogenic bacteria, protection from mastitis infection in nonlactating udders, and opsonic activity and immunoregulatory function (i.e., contribution to the preimmune innate defense of mammals) (Ekstrand, 1994; Naidu, 2000, 2002). LF possesses antioxidant properties and has a broad antimicrobial spectrum including antibacterial, antifungal, antiprotozoal, antiviral, and antitumor properties (Naidu, 2000).

Lactoferricin (LFcin) is an active peptide that is derived from peptic hydrolysis of human and bovine LF close to the N-terminus (Jones et al., 1994; Tomita et al., 1994). It exhibits marked antimicrobial effects and, in a majority of studies, is more efficacious than LF. Activated LF (ALF)

is an immobilized form of LF on edible substrates and was developed as a deterrent to pathogenic bacteria that may be present on meat surfaces (Naidu et al., 2003). Specifically, ALF is presented to act as a blocking agent, which interferes with microbial adhesion/colonization, detaches live or dead microorganisms from biological surfaces, inhibits microbial growth/multiplication, and neutralizes the activity of endotoxins (Naidu, 2002). It is considered a GRAS compound by the U.S. Food and Drug Administration (21 CFR.170.36[f]) because it is a derivative of the naturally occurring LF in milk, which in turn is isolated from cheese-whey and skim milk of bovine origin. Use of ALF on fresh beef was approved (October 2001) by the U.S. Department of Agriculture to prevent bacterial contamination during processing (Naidu et al., 2003).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

LF is present at 20 to 200 mg/L and >2000 mg/L in bovine and human milk, respectively, as well as two major reservoirs of mammals, including a circulatory pool in the secondary (specific) granules of polymorphonuclear neutrophils at 15 to 30 $\mu\text{g}/10^7$ cells, depending on the age of the individual (Baggiolini et al., 1970; Baveye et al., 1999; Bennet and Kokocinski, 1978; Levay and Viljoen, 1995; Naidu, 2000), and a stationary pool on the mucosal surfaces (van Hooijdonk et al., 2000; Levy, 1996; Levay and Viljoen, 1995; Lonerdal and Lyer, 1995). The presence of LF in mucosal surfaces originates from epithelial cells or submucosal glands that excrete LF by the influence of the parasympathetic nervous system (Naidu, 2000; Raphael et al., 1989; Testa, 2002). Examples of mucosal surfaces where LF can be found are lactating breast tissues, gastric tissues, and duodenal epithelial cells (Mason and Taylor, 1978). LF accounts for 11.5% of the total secretory proteins excreted by bronchial glands (Harbitz et al., 1984) and 25% of the total tear protein produced by lachrymal glands (Kijlstra et al., 1983). In addition, concentrations of 0.2 to 1.0 mg/ml are found in seminal plasma (Witchman et al., 1989) and have been found in the sperm-coating antigen (Ashorn et al., 1986).

The highest levels of LF (5 to 7 g/L) are observed in colostrum and gradually decrease by almost 7-fold in mature milk during lactation (Hennart et al., 1991; Hirai et al., 1990; Levay and Viljoen, 1995; Lönnerdal and Iyer, 1995; Montagne et al., 1998; Playford et al., 2000). In plasma it is normally found at low levels (approximately 0.2 to 1.6 $\mu\text{g}/\text{ml}$) (Levay and Viljoen, 1995; Steijns and van Hooijdonk, 2000; van der Strate et al., 1999). Milk from mastitic cows contains higher levels of LF than that from healthy ones. In addition, the concentration of LF is higher when infection is caused by *Staphylococcus aureus* and streptococci species rather than when caused by coagulase-negative staphylococci or *Corynebacterium bovis* (Hagiwara et al., 2003; Kai et al., 2002), indicating the antimicrobial role of the compound and its potential to prevent disease.

Increase of LF in plasma may occur during acute-phase host responses, such as infection, inflammation, or toxic shock (Klasing, 1984; Baynes et al., 1986; Naidu, 2000; Qadri et al., 2002). Van der Strate et al. (1999) established a linear correlation between concentrations of LF in plasma and counts of neutrophils. Elevated levels of LF in plasma is a primary indication of inflammatory state, such as septicemia and endotoxemia (Gutteberg et al., 1988), and is commonly followed by an increase of LF in serum, thus making LF levels a reliable diagnostic tool for pathologic conditions (Qadri et al., 2002). Increases of LF both in plasma and serum also correlate with increases in C-reactive protein during meningococcal septicemia (Gutteberg et al., 1984), cystic fibrosis protein during cystic fibrosis (Barthe et al., 1989), cholecystokinin secretion during pancreatitis (Dite et al., 1989), or lysozyme during localized juvenile periodontitis (Friedman et al., 1983). The iron-chelating properties of LF are an additional reason for increased clinical levels of LF (e.g., during rheumatoid arthritis) (Blake et al., 1981, 1984; Bukhardt and Schwingel, 1986) as well as in various pathogenetic malignancies (especially carcinomas), when iron uptake by neoplastic cells is necessary (Barresi and Tuccari, 1987).

Isolation and Purification

Naidu (2000) reviewed the reported methods for isolation of LF from mammalian milk and indicated that the most commonly used are affinity chromatography on CM-Sephadex, Cibachron Blue-Sepharose, heparin-cross-linked and DNA-agarose columns. In addition to these methods, Ye et al. (2000) proposed a rapid ion-exchange chromatography method for isolation of cow milk proteins, including LF, that involves sequential passage of milk through a strong cationic- (sulphopropyl-Toyopearl) and a strong anionic-exchanger (quaternary aminoethyl-Toyopearl). In 2002, a cation-exchange chromatographic method was developed and used to evaluate the purity and N-terminal integrity of human LF (van Veen et al., 2002). Currently, LF is isolated and purified on an industrial scale at approximately 20 to 30 tons annually worldwide from cheese-whey and milk (Tomita et al., 2002). The concentration of LF in cheese-whey is roughly 100 mg/L, and because of its cationic nature, it is easily adsorbed to a cation-exchange resin that may be eluted using salt solutions. Purification (to 95% purity) occurs *via* ultra-filtration (or microfiltration) with diafiltration membranes followed by pasteurization at 90°C to 100°C for 5 to 10 minutes (pH 4.0) and freeze-drying or spray-drying (Tomita et al., 2002).

The production technology (Naidu, 2001) of ALF is based on the immobilization of milk LF via its N-terminus region on a food-grade glycosaminoglycan such as galactose-rich polysaccharide or carrageenan, with elimination of the factors that are reported to decrease or reverse the activity of LF. Therefore, immobilization is accompanied by neutralization of cationic peptides by salt, optimization of substrate conditions through adjustment of pH and citrate/bicarbonate ratio, and also establishment of an equilibrium between bound (immobilized) and unbound LF. Similarly, these steps are necessary for *in vitro* activation of LF (Naidu, 2001).

Physicochemistry

LF is a single polypeptide chain with a molecular weight of 75 to 80 kDa and consists of approximately 690 amino acid residues (Baker et al., 2000; Metz-Boutigue et al., 1984; Powel and Ogden, 1990; Rey et al., 1990). The amino acid composition of LF includes 16 to 18 different amino acids (of which 8 are basic); the frequency and types of amino acids depend on the mammalian species (Naidu, 2000).

The reported isoelectric point (pI value) for LF varies with the measuring method. According to electrophoretic, chromatofocusing, and Rotafors methods, the pI of LF is around 8.0 (Groves, 1960; Shimazaki et al., 1993; Szuchet-Drechin and Johnson, 1965), although it may range from 5.5 to 10.0 (Naidu, 2000). In general, LF is considered a basic protein (with an expected pI higher than 7.0) because its N-terminal region contains multiple arginine (arg) and lysine (lys) residues that make this region extremely basic (Vogel et al., 2002). The heat resistance (known to be stable at 90°C for 60 minutes) and heat-induced enthalpy of LF change depend on its iron-binding status and pH of the substrate. The iron-saturated form of LF (i.e., holo-form [$87 \pm 3^\circ\text{C}$]) is more stable than the nonsaturated form (i.e., apo-form [$67 \pm 3^\circ\text{C}$]) (Naidu, 2000; Paulsson et al., 1993; Reiter and Oram, 1967; Rossi et al., 2002). Neutral and basic pH enhances thermal denaturation of LF, whereas LF is more heat-stable at low pH, especially around 4.0 (Saito et al., 1994). The holo-form of LF is more resistant to proteolysis than the apo-form (Brock et al., 1976). Moreover, binding of Ca^{2+} via the carboxylate groups of the protein surface increases the chemical and thermal stability of bovine LF (Rossi et al., 2002). Specifically, the presence of Ca^{2+} causes an approximate 9°C increase in the denaturation temperature of both the holo- and apo-form of LF (Rossi et al., 2002).

Structure

LF is characterized by an amphipathic structure with strong cationic properties on the N-terminus (Bellamy et al., 1992). The three-dimensional structures of human and bovine LF have been determined to a 2.8 Å resolution (Naidu, 2000; Thakurta et al., 2003), and a high degree of homology

of this protein has been observed between species. LF is folded into similarly sized globular and homologous terminal N and C lobes, which are stabilized by intrachain disulfide bonds and linked by an α -helix that provides flexibility (Baker et al., 1987; Vorland, 1999; Baveye et al., 1999; Naidu, 2000). The symmetry of the lobes derives from gene duplication, and they are further divided into two similar domains of about 160 amino acids (Testa, 2002). Each lobe contains glycosylation sites (the number varies among mammalian species) in homologous position and has the capacity to bind one Fe^{3+} with high affinity ($K_d = 10^{20} \text{ M}^{-1}$) together with one CO_3^{2-} or HCO_3^- ion that is held electrostatically to an arginyl side group (Naidu, 2000). Each iron atom is coordinated to four protein ligands in accordance with the carbonate or bicarbonate ion. Certain areas within the molecular structure serve as potential cavities for binding not only of carbonate but also of larger anions. Specifically, the HCO_3^- occupies a pocket between the iron and two positively charged groups and serves to neutralize this positive charge, thus facilitating binding of iron ions. Using X-ray structure analysis it has been concluded that the multidomain nature and the flexibility of LF allow for binding of various metals with no significant changes in the total structure (Naidu, 2000). Apart from the sites for iron absorption, metal binding sites for other cations, such as Cu(II), Cr(III), Mn(III), and Co(III), are available within the structure of LF similarly to serum transferrin (Ainscough et al., 1980; Hirose, 2000; Naidu, 2000). The anion-binding properties of LF are affected by the type of metal ion that is already bound. Anions that have been investigated for their potential to bind LF in the presence of Fe^{2+} or Cu^{2+} include carbonate, citrate, oxalate, and hybrid carbonate–oxalate complexes (Brodie et al., 1994). The role of citrate/bicarbonate ratio is crucial for the apo- to holo-form transition of LF (Nonnecke and Smith, 1984), which controls the iron-binding process and hence the biological activity of LF.

LFcin is produced by peptic hydrolysis (also mentioned as cleavage degradation) of bovine or human LF (Bellamy et al., 1992; Tomita et al., 1994) with different proteases. Bovine LFcin (commonly termed “lactoferricin B,” or LfcinB), was found to consist of 25 amino acid corresponding to the sequence of residues 17 to 41 close to the N-terminal of the molecule (Pierce et al., 1991; Tomita et al., 1994; Wakabayashi et al., 1992). Human LFcin (commonly termed “lactoferricin H,” or LfcinH) was found to consist of 47 amino acid residues, corresponding to the sequence of residues 1 to 47 at the N-terminal of the human LF (Bellamy et al., 1992; Metz-Boutigue et al., 1984). Both LfcinB and H have an almost circular structure consisting of a loop of 18 amino acid residues that links two linear subfragments and have molecular weights of 3126 and 5558, respectively (Bellamy et al., 1992; Metz-Boutigue et al., 1984; Pierce et al., 1991). The linkage of subfragments occurs through a disulfide bond between the two terminus cysteine residues of the loop (Bellamy et al., 1992).

ANTIMICROBIAL ACTIVITY

Mode of Action

The diversity of substrates in which LF is present, its coexistence with other physiologic substances, and the regulatory role of LF as acute-phase reactants explain its multifunctionality and its wide spectrum of antimicrobial activity (van Hooijdonk et al., 2000; Naidu, 2000). It is considered that the antimicrobial activity of LF is generally dependent on its protein conformation (structural characteristics and spatial orientation) and substrate conditions (Naidu and Arnold, 1997). In particular, two basic biochemical properties of LF contribute to its antimicrobial effect and involvement in the host defense: the extremely powerful iron-binding capability and its strong interaction with other molecules and surfaces. The amphipathic structure and the net positive charge, especially on the strongly cationic N-terminus region of LF, are considered deterministic for its interaction with microbial membranes (Bellamy et al., 1992). Binding of LF occurs via specific membrane receptors that exist, for example, in the leukemic lines, the activated T-lymphocytes, the monocytes–macrophages, the brush-border membranes, the parenchymal liver cells, the breast epithelial

cells, the platelets, and the neuronal cells (Testa, 2002). Specific molecules that interact with LF are glycosaminoglycans of epithelial milieu (e.g., heparin sulfate in mucins), collagens, fibronectins, and DNA of mammalian cells (Naidu, 2002; Nichols et al., 1990; Wu et al., 1995).

As previously mentioned, LF is a broad-spectrum antimicrobial and modes of action appear to be common for different types of microorganisms. Among the studies conducted to elucidate mechanisms of antimicrobial activity, most have been performed with bacteria and viruses, although work has been done with fungi and protozoa. The following section introduces potential mechanisms by which LF may inhibit microorganisms, specifically bacteria and viruses. LF may be bacteriostatic or bactericidal, and iron deprivation is the most likely the underlying mechanism for direct bacteriostatic effect of LF (Naidu, 2000) as demonstrated by *in vitro* studies. However, bacteria such as *Escherichia coli*, *Neisseria* species, *Moraxella catarrhalis*, and *Vibrio* species have potential defensive mechanisms to counteract iron depletion, mainly by formation of siderophores that mediate iron uptake. Alternatively nonsiderophore-mediated iron uptake by bacteria may occur either through outer-membrane protein receptors (*Neisseria meningitis*) that recognize the complex of LF iron and internalize the chelated iron cation (Mickelsen et al., 1982; Naidu, 2000; Schryvers and Morris, 1988; Tranter, 1994) or by production of reductases as is the case with *L. monocytogenes* (Coward and Foster, 1985). Moreover, the bacteriostatic activity of LF may be reduced by factors that are irrelevant to microbial resistance, such as inappropriate citrate/bicarbonate ratios, given that bicarbonate is essential for iron chelating, whereas citrate competes with LF for iron and hence makes the iron available for growth by the organisms (Reiter, 1978b).

The bactericidal activity of LF is iron-independent and relates to direct binding of positively charged LF on negatively charged microbial outer membranes, which results in dispersion of lipopolysaccharides (or negatively charged fatty acids), increase in membrane permeability, and eventually cell death (Arnold et al., 1980; Baveye et al., 1999; Caccavo et al., 2002; Ellison et al., 1988; Naidu and Arnold, 1997; Pellegrini, 2003; Rossi et al., 2002; Yamauchi et al., 1993). Ellison et al. (1990) suggested that Ca^{2+} and Mg^{2+} are the major cations that modulate the LF-induced damage in Gram-negative bacteria; this conclusion was confirmed by Rossi et al. (2002). Binding of LF to microbial cell surfaces, however, is enhanced by bacterial receptors that exist in a variety of Gram-positive bacteria (e.g., lipoteichoic or teichoic acids, surface layer proteins, peptidoglycan components, heat-shock proteins [*Helicobacter pylori*]) and Gram-negative bacteria (e.g., lipid A, whereas the pore-forming outer membrane proteins [porins] are a very common binding target of LF) (Amini et al., 1996; Baveye et al., 1999; Caccavo et al., 2002; Dhaenens et al., 1997; Erdei et al., 1994; Naidu, 2000; Naidu and Arnold, 1994; Naidu et al., 1993; Sallmann et al., 1999; Vorland et al., 1999c). Likewise, antibiotic potentiation has also been demonstrated *in vitro* as a result of an increase in permeability of outer membranes (Naidu and Arnold, 1994; Vorland et al., 1999c; Wakabayashi et al., 2002). An additional antimicrobial function that stems from the interaction of LF with the outer membrane of Gram-negative bacteria is the inhibition of microbial attachment to subepithelial matrix proteins or, alternatively, detachment of bacteria from mucosal surfaces (Kawasaki et al., 2000; Naidu and Bidlack, 1998). Blocking of cell-surface attachment factors, such as fimbriae and other adhesins, and inhibition of the colonization-factor antigens synthesis are associated with this antimicrobial mechanism (Naidu and Bidlack, 1998; Naidu, 2002). Moreover, LF binds on many tissue surfaces with higher affinity than cell-surface anchors of pathogens (Naidu, 2002). Bellamy et al. (1993) demonstrated an additional mechanism derived from membrane disruption (similar to that of polymyxin B) related to the inhibition of proline uptake. Optimal binding (dose-dependent) to membranes and biocidal effect was obtained at pH 6.0 and pH 7.5 for *E. coli* and *Bacillus subtilis*, respectively. The interaction of LF and LF_{cn} with microbial membranes has also been found to account for their fungicidal activity (Bellamy et al., 1993; Wakabayashi et al., 1996).

A more detailed investigation of the mode of action of both LF and its hydrolysates, oriented toward the effect of LF on liposomes, electrochemical potential ($\Delta\Psi$), and pH gradient of Gram-negative pathogens, has become available in literature over the last few years. Many studies aim

to fully elucidate all the potential underlying mechanisms of antimicrobial activity, as well as to detect synergism phenomena between LF and other compounds. It was shown that 0.1 to 6.4 μM of iron-free LF increased permeability of the outer and inner membrane of *E. coli*, reduced by 50% or dissipated the electrical potential and the pH gradient, and also caused selective ion permeabilization on liposomes of *E. coli* (Aguilera et al., 1999, 2003). Different amino sequences of LFcinB were found to enter the cytoplasm of *S. aureus* and *E. coli* within 15 minutes of exposure, whereas lower quantities of LFcinB were also found in the cell wall (Haukland et al., 2001). Penetration and attachment to cell wall was proved to be time- and concentration-dependent. Ulvatne et al. (2001) illustrated that 30 to 100 $\mu\text{g}/\text{ml}$ of LFcinB, corresponding to concentrations below the minimum inhibitory concentrations (MIC) or minimum bactericidal concentrations (MBC), caused morphologic changes of cells and depolarized the cytoplasmic membrane and destabilized liposomes, causing leakage and fusion of liposome contents, whereas no cell lysis was observed. These indications emphasize that membrane destabilization is the major mechanism for the antimicrobial activity of LFcinB.

Investigation of the synergism between LFcinB and antibiotics has provided useful indications about potential clinical applications as well as for elucidation of the mode of action of LFcinB and/or common antibiotics. The antimicrobial effect of antibiotics, which are usually excluded by the outer membrane of Gram-negative bacteria (e.g., erythromycin, vancomycin, and penicillin), may be enhanced by LFcin, which destabilized bacterial membranes and facilitated their penetration (Diarra et al., 2002; Vorland et al., 1999b). Other antibiotics, such as polymyxins, which act on membranes, may compete with LFcin with a moderated antimicrobial effect. Similarly, prevention of entry of ribosome targeting aminoglycosides (gentamycin) into cells as a result of disturbance in the respiratory chain of the cytoplasmic membrane caused by LFcinB may also result in reduction of the antimicrobial effect of antibiotics (Vorland et al., 1999a). For the previously outlined reasons, LFcinB acted synergistically with penicillin against *S. aureus* and with erythromycin against *E. coli*, whereas antagonism was evident between gentamicin and LFcinB against *S. aureus* (Vorland et al., 1999a). In another study, microcyclin and other compounds (e.g., acids, alcohols, and acylglycerols) enhanced the antimicrobial activity of LFcinB against antibiotic resistant strains of *S. aureus* (Wakabayashi et al., 2002). The effect of sequence of application of LFcin and antibiotics was studied *in vitro* by Haukland and Vorland (2001), who suggested that LFcinB had no overwhelming effect when applied at postantibiotic stage against *E. coli* and *S. aureus*. Microbial resistance to LF is a potential response and a possible resistance mechanism for *E. coli*, and *S. aureus* involves production of proteases that decompose LFcin (Ulvatne et al., 2002). Additionally, the presence of Mg^{2+} ions in the growth environment of cells prior or during exposure to LFcin may also offer protection against subsequent exposure to LFcin by altering the properties of the outer membrane (Masschalk et al., 2003).

With respect to antiviral activity of LF, according to Naidu and Bidlack (1998) and van der Strate et al. (2001), the potential mechanisms for antiviral activity are the following: (1) prevention of viral infection by direct binding of LF to virus particles (e.g., envelope proteins); (2) interference with virus docking into cells by binding of LF either to sulfate proteoglycans (HSPGs) or to viral receptors of the host cells that are used by virus for intracellular entry; (3) inhibition of virus proliferation; (4) intracellular activity of LF likely associated with interference with antigen synthesis of virus (e.g., during infection by rotavirus); and (5) other indirect mechanisms, such as cell cytopathy, regulatory function of LF on myelopoiesis, and cytopathy during viral infection (e.g., friend virus complex, inhibition of viral hemagglutination, like human influenza virus). Factors such as the stage of infection (i.e., duration of infection before addition of LF) (Vorland, 1999); other synergistically acting compounds, such as zidovudine (Viani et al., 1999); and the saturation of LF with different metals (e.g., Zn^{2+} , Mn^{2+} , Fe^{3+}) are crucial for the antiviral outcome of LF (van der Strate et al., 2001).

Finally, LF is important in immunoregulation and specifically in the first line of the host defense system, which is an interaction of neutrophils, lymphocytes, macrophages, and their secretory

products. Synergism with antibodies, or activation of a complex series of reactions, is associated with the contribution of LF to the overall protective immune response after infection or inflammation (Levay and Viljoen, 1995; Lönnerdal and Iyer, 1995; Sanchez et al., 1992; Machnicki et al., 1993; Ward et al., 2002; Zagulski et al., 1989). Specifically, when contamination with a pathogen occurs, the polymorphonuclear neutrophils capture the invader (phagocytosis) and specific granules release low-iron-saturated LF (6% to 8%) into the blood, which in turn creates a hypoferremic environment (depletion of iron), thus preventing the pathogen from acquiring sufficient iron for growth (Miyachi et al., 1998; Sanchez et al., 1992; Ward et al., 2002). Affinity of LF to lipopolysaccharides (LPS) is also beneficial because binding of LF to lipid A, the toxin moiety of LPS of Gram-negative pathogens, prevents the toxic shock induced by interaction of this lipid with monocytes/macrophages (Baveye et al., 1999; Caccavo et al., 2002). A review of mechanisms related to modulation and amplification of the inflammatory process by LF that was presented by Baveye et al. (1999) listed the following mechanisms: (1) enhancement of phagocytosis by preventing the deactivation of complement factor C3; (2) inhibition of extracellularly formed hydroxy-radicals by sequestration of free iron; (3) enhancement of polymorphonucleocytes (PMNs) recruitment to inflammatory sites; (4) regulation of the proliferation and differentiation of immune cells (suppression of myelopoiesis) by suppressing the production of interleukins IL-1 and IL-6 by monocytes; (5) inhibition of platelet aggregation; and (6) promotion of the recruitment and activation of immune cells in inflammatory sites by blocking the release of other cytokines (e.g., the tumor necrosis factor alpha, or TNF- α) (Crouch et al., 1992; van Hooijdonk et al., 2000; Machnicki et al., 1993; Mattsby-Baltzer et al., 1996; Shinoda et al., 1996; Vorland, 1999; Wagstrom et al., 2000; Zagulski et al., 1989). A number of studies reviewed by Ward et al. (2002) illustrated that inhibition of TNF- α by LF may protect human and mice from allergen-induced skin inflammation. Moreover, it is shown that LFCin and some of its acylated derivatives inhibit the lipid peroxidation in liposomes (Wakabayashi et al., 1999a). In one of the latest reviews on LF, Wakabayashi et al. (2003) used terms such as immunosuppression and immunostimulation to describe the previously mentioned immunoregulatory activities of LF and LFCin or its derivatives with shorter amino acid sequences. Immunostimulation and specifically the stimulation of phagocytic and cytotoxic properties of macrophages were also termed opsonic effects (Naidu, 2000). A list of major binding targets of these peptides includes LPS, heparin, DNA, glycosaminoglycan, etc., and some more specific mechanisms, such as induction of apoptosis in monocytic and myeloid leukemic cells, or activation of kinase CK2, commonly representative of different LFCin residues (Maekawa et al., 2002; Roy et al., 2002; Yang et al., 2002).

ALF as an immobilized derivative of LF possesses identical mechanisms for antimicrobial activity, summarized into three major categories: (1) blocking of microbial adhesion; (2) bacterial detachment; and (3) microbial growth inhibition (Naidu, 2002). Interaction of ALF with the outer membranes of bacteria, interfering with adhesin/fimbrial synthesis or colonization factors and competing with bacteria in adhesion on tissue-matrix components, are the properties responsible for the first two of the previously listed mechanisms. Iron deprivation with further consequences on ATP synthesis and cellular multiplication accounts for the growth inhibitory mechanism, and interaction of ALF with nucleic acid is likely associated with its antiviral activity.

Specificity

LF is known to possess a wide antimicrobial spectrum including Gram-positive and Gram-negative bacteria, such as *Helicobacter pylori*, *E. coli* O157:H7, *E. coli* O111, *B. subtilis*, *S. aureus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *P. aeruginosa*, *L. monocytogenes*, *Micrococcus flavus*, *Salmonella* Typhimurium (Bellamy et al., 1993; Naidu, 2000; Viejo-Diaz et al., 2003; Wakabayashi et al., 2003), yeast of the genus *Candida* species (Kuipers et al., 2002; Samaranyake et al., 1997; Ueta et al., 2001; Wakabayashi et al., 1996, 1998), fungus such as *Rhodotorula rubra* (Andersson et al., 2000), *Penicillium* species (Liceaga-Gesualdo et al., 2001), *Trichophyton* species (Wakabayashi et al., 2000), RNA and DNA, enveloped or nonenveloped viruses (Naidu, 2000; van der Strate,

2001; Vorland, 1999), and protozoa including *Toxoplasma gondii* (Isamida et al., 1998), *Giardia lamblia* (Turchany et al., 1995), and *Trichomonas foetus* (Grab et al., 2001).

For determination of MIC and MBC of bovine and human LF as well as their hydrolysates (i.e., LFCinB and LFCinH), the standard microdilution method of Vorland et al. (1999b) in 1% peptone water has been used by the majority of researchers. In general, hydrolysates of LF (i.e., LFCins) and especially LFCinB are more inhibitory than the original LF (Naidu, 2000). It is important to note that LFCin of human origin is more active than LFCin of bovine, murine, and caprine origin (Vorland et al., 1998). According to Bellamy et al. (1992), who performed a screening on MIC of bovine and human LF as well as the corresponding LFCins against *E. coli* O111, the MICs of human and bovine LF were 2000 µg/ml and 3000 µg/ml (37µM), respectively, whereas the MIC of LFCinH was 100 µg/ml (ca. 10-fold lower than that of LF), and that of LFCinB was 6 µg/ml (almost 1000-fold lower than LF). In the same review, the MIC of LFCinB was found lower than the MIC of bovine LF against a variety of other microorganisms, including *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes*. In a more recent study, Kimura et al. (2000) reported that MIC of Korean goat LF against *E. coli* O111 was 5000 µg/ml (i.e., higher than that of bovine LF but lower than the MIC of sheep and horse LFs) (Lee et al., 1997). The results of this study also agree with the observed differences between the antimicrobial activity of bovine LF and its hydrolysate because the MIC of Korean goat LFCin was only 100 µg/ml (i.e., similar to that of LFCinH and 50-fold lower than that of Korean goat LF). Ulvatne et al. (2001) demonstrated that *P. mirabilis* was more resistant to LFCinB than *E. coli*.

In a study by Recio and Visser (2000), the antibacterial effect of apo- and holo-form of bovine, ovine, and caprine LF was comparatively evaluated in a medium containing 1% peptone and 1% glucose, against *E. coli* and *M. flavus*. Of the apo-lactoferrins, caprine LFCin had the highest effectiveness against both microorganisms, followed by bovine and then ovine LF. Holo-forms of LF were almost ineffective. Griffiths et al. (2003) comparatively investigated the effect of apo-form and the holo-form bovine and human LF against *E. coli* and *S. Typhimurium* in coculture with probiotic bacteria, such as bifidobacteria. A conclusion of high importance for the protective role of intestinal flora was that apo-form of LF retarded growth of enteric pathogens without affecting the growth of probiotic bacteria. This conclusion is beneficial for the potential application of LF in foods compared to antibiotics, given the deleterious effect of the latter to intestinal flora of humans.

The antimicrobial effect of several LFCinB analogs (i.e., peptides with shorter amino acid sequences) has also been considered by many research groups with varied results related to their effectiveness compared to the original LFCinB (Chapple et al., 1998; Chen et al., 2003; Hoek et al., 1997; Kang et al., 1996; Odell et al., 1996; Rekdal et al., 1999; Strom et al., 2002a,b; Ueta et al., 2001; Wimley and White, 2000). Schibli et al. (1999) reported that the 6-residue center of LFCinB is responsible for the antimicrobial activity of the whole molecule. Usually, the examined analogs are 11- or 15-residue LFCin derivatives synthesized by incorporation of certain amino acids on residues 6 and 8 (for addition of tryptophane), 5 and 9 (for addition of arginine), or other residues (e.g., lysine) of LFCin (Chen et al., 2003; Haug and Svendsen, 2001; Kang et al., 1996; Strom et al., 2000, 2002a,b). The antimicrobial activity of these analogs depends on the type of incorporated amino acids, the terminus of LFCin where addition of amino acids is made (N-terminal analogs show higher variability on antimicrobial effectiveness than C-terminus analogs), as well as the charge and the lipophilicity of the resulting analog (Strom et al., 2000, 2002). Tryptophane derivatives seem to be the most effective analogs, probably as a result of high membrane affinity of this amino acid (Haug and Svendsen, 2001; Strom et al., 2002b; Wimley and White, 2000).

Wakabayashi et al. (1999b) demonstrated that N-acylated and D-enantiomer derivatives of LFCin possess increased antibacterial and antifungal activity as indicated by the higher MICs of LFCinB. The MICs of these derivatives ranged from 3 to 12 µg/ml against bacteria, such as *E. coli*, *P. aeruginosa*, *S. aureus*, and the fungus *Trichophyton mentagrophytes*, whereas significantly higher MICs, ranging from 25 to 100 µg/ml, were observed for *C. albicans*. Vorland et al. (1999a) made

a comparative test on the MIC of LFCin and its peptides synthesized by L- and D- amino acids, on spheroplasts of *E. coli*, *P. mirabilis*, and protoplasts of *S. aureus* as well as on the previously mentioned microorganisms with intact cell walls. The D-enantiomer of LFCin was more active than the L-enantiomer. LFCin (MIC 30 $\mu\text{g/ml}$ and MBC 80 $\mu\text{g/ml}$) and its D-enantiomer (MIC and MBC equal to 30 $\mu\text{g/ml}$) had the same effect on *S. aureus* but the D-enantiomer, with MIC and MBC equal to 5 $\mu\text{g/ml}$, was far more effective than LFCin against *E. coli*. None of the tested peptides was effective against *P. mirabilis*. Spheroplasts and protoplasts were more sensitive than their counterparts, suggesting that the cell wall has a protective role against LFCin. Lower temperatures (22°C and 28°C) sensitized bacteria to all tested peptides in comparison with 37°C; similar results for the effect of the D-amino acid counterpart of LFCin were reported by Ulvatne and Vorland (2001).

The antifungal properties of LF and LFCins have also been investigated. Wakabayashi et al. (1996) reviewed the antifungal spectrum and fungicidal mechanism of LFCinB. *Candida* species, as a result of their clinical significance as the causative agents of many types of candidosis, are the most extensively used organisms in studies for investigation of antifungal effect of LF and LFCins. Indeed, the antifungal effect of LF, LFCin, and LFCin residues alone (Xu et al., 1999) or in combination with other compounds (e.g., azole antifungal agents) (Wakabayashi et al., 1996, 1998), or in the form of sodium alginate tablets (Kuipers et al., 2002), against *C. albicans* (Ueta et al., 2001) and other species (e.g., *C. glabrata* or *C. krusei*) has been well established (Samaranayake et al., 1997). Inhibitory effect has also been reported for other fungi (Wakabayashi et al., 2003), including *Rhodotorula rubra* (Andersson et al., 2000), spores of *Penicillium* species (Liceaga et al., 2001), and *Trichophyton* species (Wakabayashi et al., 2000).

Additionally, LF and its hydrolysates have been shown *in vivo* (in mice) to inhibit protozoa, such as *Toxoplasma gondii* (Isamida et al., 1998), *Giardia lamblia* (Turchany et al., 1995), and tumour metastasis (Yang et al., 2002; Yoo et al., 1997). Some protozoa, however, such as *Tritrichomonas foetus*, are reported to sequester the iron necessary for survival from the host (cattle) LF (Grab et al., 2001). Moreover, LF has antiviral activity against a wide range of human and animal RNA and DNA viruses, enveloped or not (Naidu, 2000; van der Strate, 2001; Vorland, 1999), such as hepatitis C virus (Ikeda et al., 1998, 2000), cytomegalovirus (Andersen et al., 2001), rotavirus (Superti et al., 1997), friend virus (Vorland, 1999), poliovirus (Marchetti et al., 1999), respiratory syncytial virus (Grover et al., 1997), human immunodeficiency virus (Swart et al., 1996, 1998; Vorland, 1999), human influenza virus (Kawasaki et al., 1993), spleen focus forming virus (Hangoc et al., 1987), feline immunodeficiency virus (Sato et al., 1996), and herpes simplex virus (Hammer et al., 2000; Hasegawa et al., 1994). In contrast, LFCin does not always demonstrate antiviral activity (Ikeda et al., 2000).

APPLICATIONS IN FOODS

LF is available in ready-to-use form such as liquid or spray-dried powder. In the past two decades, its metal-chelating property has been the primary claim for the application of LF in several infant food formulas in southeast Asian markets (Satue-Gracia et al., 2000). Nandi et al. (2002) successfully induced expression of LF in transgenic rice grains at levels up to 0.5% by linking a synthetic LF gene to rice glutelin 1 promoter for future application in infant formulas. The pharmaceutical application of LF and its hydrolysates LFCinB and LFCinH are also well established (Clare et al., 2000, 2003; Yamaoto et al., 2003), as are their potential benefits in curing infected fish and seafood (Kakuta, 2000; Koshio et al., 2000; Gallardo-Cigarroa et al., 2000). Oral administration of bovine LF is reported to enhance survival of rainbow trout, red sea bream, and goldfish on infection with *Aeromonas salmonicida*, *Cryptocaryon irritans*, and *Ichthyophthirius multifiliis*, respectively (Kakuta, 2000). However, the application of these compounds in foods is still limited so far as a result of the requirement of high doses to obtain a preservative effect. For instance, susceptibility of LF to pH, elevated levels of calcium or phosphates, excess of cations (especially iron), and improper citrate/bicarbonate ratios are factors with the potential to decrease the activity of LF

(Naidu, 2002). The antimicrobial activity may also be reversed by trypsin, ferrous sulfate, magnesium sulfate, and hematin (Reiter and Oram, 1967). In addition, phenomena related to the risk of denaturation, structural alterations, or even charge-induced aggregation during isolation of LF pose further limitations in the application of lactoferrin in foods. Therefore, research has been focused on discovery of alternatives to overcome the limitations in application of LF. Potential alternatives include the following: (1) activation of LF under conditions that protect its structure and minimize the negative impact of milieu conditions, thus resulting in activated lactoferrin; and (2) LF digestion derivatives (i.e., LFs and their analogs with smaller amino acid residues in an effort to evaluate the potential of compounds, which are released naturally as a result of proteolysis of LF and LFCin, respectively).

Investigation of antimicrobial properties of LF and LFCins in foods is still in its infancy, with a few published studies available. A common conclusion drawn from these studies is that the activity of LF is reduced as a result of the excess of metal cation that saturates the peptide. Thus, the presence of other compounds with chelating properties, such as ethylenediamine tetraacetic acid (EDTA), or dilution of cations concentrations are necessary to enhance LF activity (Branen and Davidson, 2000; Chantaysakorn and Richter, 2000; Masschalck et al., 2003). Alternatively, combination of LFCin with other emerging technologies, such as high hydrostatic pressure, has proved to be promising, especially when prevention of saturation of LFCin with ions is not feasible (Masschalck et al., 2001, 2003).

LFCinB was primarily tested in ground beef at concentrations of 50 or 100 $\mu\text{g/ml}$, where it was found to cause a maximum of 2 \log_{10} CFU/g reduction at 4°C or 10°C (Venkitanarayanan et al., 1999). The positive contribution of EDTA on the antimicrobial activity of LFCin was demonstrated by Branen and Davidson (2000) against *E. coli* and *L. monocytogenes* in tryptic soy broth and in a medium containing 1% peptone, 0.05% yeast extract, and 1% glucose. LFCinB at concentrations of 1600 $\mu\text{g/ml}$ was unable to inhibit growth of *E. coli* at 37°C, whereas the addition of 100 or 400 $\mu\text{g/ml}$ EDTA, depending on the strain of *E. coli*, totally prevented growth. Similar results were obtained against *L. monocytogenes*; however, lower concentrations of LFCin were necessary for inhibition as a result of the higher sensitivity of *L. monocytogenes* compared to *E. coli*. However, there seems to be a dispute on whether EDTA enhances LF activity in foods. Murdock and Matthews (2002) found no antimicrobial effect by the combination of the two compounds, in ultrahigh temperature (UHT) milk acidified to pH 4.0 and stored at 37°C, even at higher concentrations of LFCin and EDTA (i.e., 4000 $\mu\text{g/ml}$ and 10 mg/ml, respectively). With respect to the increase of LFCin activity as a result of reduced cation concentration, Chantaysakorn and Richter (2000) found that 5000 and 10,000 $\mu\text{g/ml}$ of LFCin had no effect on *E. coli* in carrot juice but significantly retarded growth when added in filtrate of carrot juice through 500- or 10,000-Da molecular weight rejection membranes and totally inhibited growth in dialysate of the filtrate. Considering the decrease in the concentration of cations in filtrate and more in dialysate of carrot juice, there is a likely negative correlation between antimicrobial activity and metal cations.

Emerging preservation technologies, such as high hydrostatic pressure when combined with LF, have proved promising in the effort to overcome the limitations in its application (Masschalck et al., 2001, 2003). High pressure (155 to 400 mPa) was reported to enhance bactericidal activity of bovine LF and LFCinB against *E. coli*, *Salmonella* Enteritidis, *S. Typhimurium*, *Shigella sonnei*, *Shigella flexneri*, *P. fluorescens*, and *S. aureus* in potassium phosphate buffer at 20°C (Masschalck et al., 2001). High pressure up to 300 mPa as a single treatment caused 1 to 2 \log_{10} reductions, whereas when applied in combination with the antimicrobial peptides, approximately 2 additional \log_{10} reductions were sustained for all microorganisms. LFCinB caused consistently higher reductions than bovine LF, apart from the combination with 400 mPa, where bovine LF indicated higher reductions than LFCinB. Recently, the same group found that combination of 100 or 270 mPa for 15 minutes with 20 $\mu\text{g/ml}$ of LFCin in phosphate buffer increased reduction *S. Typhimurium* and *P. aeruginosa* by 1 to 2 \log_{10} and 3 to 5 \log_{10} (depending on strain) in comparison with the single application of high pressure and LFCinB, respectively.

ALF is a novel LF derivative that has gained increasing interest as an alternative of LF, as a result of the limitations in application of the latter in foods. However, because ALF technology was only recently established (Naidu, 2001), there is still limited information on the efficacy of this compound in foods. In a review by Naidu (2002), preliminary research data related to the detachment efficacy and bacteriostatic effect of ALF against *E. coli* in broth, beefsteaks, and fresh beef are shown. ALF and LF at a concentration of 1% were comparatively evaluated for their efficacy to detach collagen-bound *E. coli* and delay its growth. The ALF had a 2.7-log higher detachment efficacy than LF and caused 17.4 hours more stasis on *E. coli* than LF according to impedimetric data in tryptic soy broth. Accordingly, MIC of ALF and LF was 62 µg/ml and >1000 µg/ml, respectively. Similar results demonstrating the better bacteriostatic performance of ALF compared to LF were obtained against 4 log contamination of *E. coli* on beef steaks. ALF was also tested in combination with current meat decontamination intervention strategies (cold/hot water and/or organic acids) for its efficacy to detach *E. coli* from fresh beef. Specifically, a set of sequential decontamination treatments (i.e., cold water for 10 seconds, hot water at 82°C for 30 seconds, and 2% lactic acid for 10 seconds) was applied alone or followed by spraying of 1% solution of ALF for 10 seconds to detach an approximate *E. coli* population of 7 logs. The regular sanitizing assembly alone resulted in 72.2% detachment of *E. coli* per gram of beef tissue, whereas additional spraying with ALF increased the detachment efficacy to almost 100% per g of beef tissue. ALF was deposited on meat surface as a fine mist created by electrostatic or high-pressure liquid spray nozzles in a flow adjusted by digital controllers. ALF has also demonstrated activity against other pathogenic microorganisms, such as *L. monocytogenes*, *Salmonella* species, and some meat spoilage organisms including *Pseudomonas* species and *Klebsiella* species. Sensory evaluation of strip loins treated with ALF, stored under vacuum below 3.3°C, and periodically exposed to retail display within a period of 35 days showed that ALF might extend retail display life by 1.7 to 2.5 days. This conclusion was based on evaluation of lean and fat color, percentage discoloration, and overall appearance of strip loin samples treated with ALF compared with nontreated samples.

Furthermore, Ransom et al. (2003) comparatively evaluated the effect of single or sequential dipping into solutions of ALF, LF, and lactic acid (2%) on survival and growth of *E. coli* O157:H7, *L. monocytogenes*, and *S. Typhimurium*, inoculated predipping or postdipping, on vacuum-packaged bologna at 10°C for 33 days, as well as on beef plates, beef carcasses adipose, and beef lean tissues stored aerobically at 12°C for 2 to 29 days. It was shown that dipping beef bologna slices into solutions of ALF after inoculation enhanced inactivation of *E. coli* O157:H7 and inhibited growth of *L. monocytogenes* compared to untreated slices after 33 days of vacuum-packaged storage at 10°C. Treatment with ALF before inoculation delayed growth of *S. Typhimurium*, whereas postinoculation treatment prevented growth during storage; however, dipping into LF did not affect growth of *S. Typhimurium* and *L. monocytogenes* compared with samples dipped in water. Of the treatments evaluated, lactic acid was the most effective treatment in inhibiting or reducing pathogen populations on all products, followed by ALF applied either pre- or postinoculation, exclusively on bologna, whereas LF did not appear to affect survival or growth of the bacterial populations. The same study also showed that dipping into ALF followed by dipping into 2% lactic acid was the most effective treatment for decontaminating inoculated beef carcass adipose tissue.

SAFETY AND TOLERANCE

Bovine LF as a naturally occurring compound in milk has been consumed for years at levels ranging from 50 to 75 mg/day by humans of all ages (children, teens, and adults). The adverse effects of LF on iron absorption, modulation of microflora, and prevention of infection have been investigated with human studies, whereas animal studies involved acute toxicity (Nishimura, 1991), oral toxicity (Nishimura, 1997, 2000), and Ames assay (Kawai and Tanaka, 1997). No adverse effects on iron absorption, modulation of microflora, and prevention of infection are reported for daily intake of LF ranging from 0.3 to 1.0 g/kg/day for 11 days to 5 months for infants and 1.7mg/kg/day to

60mg/kg/day for single-dose study for a total of 8 weeks for adults. Likewise, no adverse effects were evident in animal studies.

The regulatory frames of LF and ALF are based on their natural occurrence in milk and consequently in beef tissue because both of them are consumed by humans. Initially, LF was characterized as GRAS by the U.S. Food and Drug Administration (FDA) (GRAS proposal), and DMV International (De Melkindustrie Veghel International) suggested that its use is considered safe in sports and functional foods at a level of 100 mg/product serving, estimating that the daily uptake of LF from that use is 1.0 g per person per day (GRN 000077). In a previous GRAS notice (GRN 000067), National Beef suggested the use of bovine milk LF as a GRAS compound in uncooked beef (carcasses, subprimals, and finished cuts of beef) as a component of an antimicrobial spray at concentrations up to 2%. The estimated daily uptake of LF from this use was 4.1 mg per person per day. However, allergenic and immunologic data related to both GRAS notices emphasized the necessity of stating the source of LF (e.g., cow milk) in the ingredient statement of the products that contain this compound to prevent consumption of these products by people that are allergic to milk or its constituents. In a recent GRAS notice (GRN 000130), ALF Ventures (the company that commercially produces and markets ALF) suggested that milk-derived LF can be considered GRAS as a component of an antimicrobial spray for dressed beef, which will subsequently be rinsed to reduce the residues of milk-derived LF, even for people that are allergic to milk; hence, disclaiming its presence in ingredients statement is no longer necessary.

Milk-derived ALF was also considered GRAS (21 CFR.170.36[f]) and permitted at levels of 65.2 mg/kg of beef (Naidu et al., 2003); accordingly, on October 2001, the U.S. Department of Agriculture approved the use of ALF on fresh beef. However, the regulatory status of ALF and LF in other countries is not clear yet (Naidu, 2002). The European Union has issued a Directive (83/417/EEC) for the use of proteins derived from milk, but LF is not included currently. Therefore, permitted use of LF in foods may only result from the consideration of LF as a milk protein (provision 79/112/EEC). Asian countries such as Japan, South Korea, and Taiwan have listed LF among other natural compounds in the List of Existing Food Additives; however, its use (in Taiwan) is limited to some specific nutritional foods under the condition “only for supplementing foods with an insufficient nutritional content and may be used in appropriate amounts according to the actual requirements” (Naidu, 2002).

OVOTRANSFERRIN

Ovotransferrin (OTF, also called conalbumin) is an iron-binding monomeric glycoprotein that constitutes at least 10% to 12% of the total egg white solids (Beuchat and Golden, 1989; Parkinson, 1966). There are many similarities between OTF and LF, and thus the following section focuses only on those characteristics unique to OTF. Isolation and purification of OTF may be accomplished using solvent fractionation and chromatographic methods (ion-exchange chromatography or metal-affinity chromatography). Structural variability in terms of molecular size, amino acid composition, and visible/ultraviolet (UV) absorption spectra of OTF is evident among different birds and/or strains (Clark et al., 1963; Itoh et al., 1979; Lush, 1961; Osuga and Feeney, 1968). Many similarities exist between all transferrin family proteins, including OTF (i.e., a total number of 680 to 700 amino acid residues subdivided into two halves, one C-lobe and one N-lobe with 35% to 40% internal homology) (Testa, 2002). Moreover, the reported homology between OTF and human LF and serum transferrin is 49% and 51%, respectively. OTF is specified by the same gene as serotransferrin but differs from the serotransferrin of the same species only in its glycan part (Testa, 2002).

Like LF, OTF reversibly binds two Fe^{3+} ions per molecule concomitantly with two bicarbonate anions. Its high affinity for iron ($\sim 10^{30} \text{ M}^{-1}$) renders the latter unavailable for bacteria and hence inhibits their growth (Tranter and Board, 1984; Valenti et al., 1983a). Therefore, stoichiometric balance of iron in the environment is essential to maintain the effectiveness of OTF (Conner, 1993;

Valenti et al., 1983b). It is well known that saturation of OTF with iron reduces its effectiveness against many Gram-negative bacteria in hen egg albumen; however, OTF remains effective against Gram-positive bacteria, including lysozyme-resistant strains, at 30°C or 39.5°C regardless of the presence or absence of iron (Tranter and Board, 1984). The antimicrobial activity of OTF is highly dependent on milieu conditions and the target organism. Alkaline pH and elevated temperature close to the physiologic temperature of birds (~40°C) enhance the antimicrobial activity of OTF (Tranter and Board, 1984).

OTF is mainly considered to have bacteriostatic activity, although there is evidence of biocidal effect independent of iron depletion against a wide range of bacteria, such as *Pseudomonas* species, *E. coli*, *S. aureus*, *Proteus* species, and *Klebsiella* species (Valenti et al., 1982, 1983a, 1984). It has been reported to extend the lag phase and reduce the growth rate of many Gram-positive and Gram-negative microorganisms, with the latter being less sensitive. Of the Gram-positive bacteria, *Bacillus* species and micrococci are the most sensitive groups (Board, 1969). Inhibitory effect against some yeasts species, such as *Candida* species, has also been observed (Valenti et al., 1983a, 1985). Despite the negative impact of iron saturation on the antimicrobial activity of OTF, the complex of OTF with other metal cations is reported to increase its antimicrobial effectiveness. Among the complexes of OTF with metal cations tested *in vitro*, the OTF-Zn²⁺ complex indicated the highest antimicrobial effectiveness (Valenti et al., 1983b).

OTF is heat sensitive, and 80% of the activity is lost with heating to 70°C to 79°C for 3 minutes (Banwart, 1979) or 60°C for 5 minutes (Tranter, 1994). However, presence of ions, such as phosphate or citrate at pH values higher than 6.0 (Nakamura and Omori, 1979), or saturation with iron in the presence of an anion, increases resistance of OTF to heat and proteolytic action and disulfide reduction by thiols (Banwart, 1979; Williams et al., 1985).

IMMUNOGLOBULINS

Immunoglobulins, or antibodies, are a complex, heterogeneous mixture of glycoproteins that are produced by plasma cells (lymphocytes or immunocytes) and are present on the B-cell membrane or secreted by plasma cells (Goldsby et al. 2000). Ig are the effectors of the immune system responsible for binding specific antigen molecules that are foreign to a host system to enable an immune response and clearance of the foreign substances and any associated harmful effects. Ig are able to recognize, bind, and occasionally neutralize bacteria, viruses, polysaccharides, nucleotides, peptides, and proteins (Bostwick et al., 2000). As a result of the diversity and great multitude of foreign molecules or antigens (Ag) in nature, there is a need for a large number of Ig to recognize and counter all the different antigens that may exist. The great diversity of Ig molecules originates from subtle structural differences in their antigen combining sites, or variable regions, accounting for unique antigen-binding specificities (Goldsby et al., 2000). The structural differences in the regions other than the antigen-combining sites, constant regions, are related to the different effector functions mediated by antibodies, such as complement activation or binding to one or more of the Ig receptors expressed on monocytes and granulocytes (Goldsby et al., 2000; Wilson and Stanfield, 1994). The main functions of Ig are as follows: (1) Ag binding–Ig binding specifically to one or more closely related antigens via the antigenic determinant (Stanfield and Wilson, 1995); and (2) effector functions (the Ig has no direct biological effect on the Ag and secondary "effector functions" are sequestered to aid in clearance of the Ag and is usually preceded by Ag binding). Effector functions may include (1) complement fixation (a group of serum proteins that participate in an enzymatic cascade that generates the cytolytic membrane attack complex and results in lysis of cells through release of biologically active molecules); and (2) binding to various cell types such as phagocytic cells, lymphocytes, platelets, mast cells, and basophils that have receptors that bind Ig and activate the cells to perform a clearance function (Stanfield and Wilson, 1995). Those functions associated with lactoglobulins and ovoglobulins are mainly complement activation and

Ig-augmented activity including agglutination, opsonization, adherence-blocking, and neutralization to aid in clearance of microorganisms.

LACTOGLOBULINS

Introduction

Colostrum, as a source for transporting immune factors including Ig from mother to newborn, has been known for more than 100 years. While colostrum remains an important source, in humans, rabbits, and rodents, passive immunity is conferred mainly through transfer of immune factors via the uterus. In contrast, for livestock (pigs, horses, sheep, and cattle) colostrum serves as the major source of immunity (Bostwick et al., 2000; Butler, 1994; Hilpert et al., 1987; Reddy et al., 1988; Stephan et al., 1990; Yolken et al., 1985). The use of commercially produced and purified Ig as antimicrobials for prevention and treatment of microbial diseases in humans is a fairly recent concept (Weiner et al., 1999) and use in food systems for preservation has been suggested (Korhonen et al., 1998; Pellegrini, 2003; Zommaro et al., 2002). The limiting factors for use of Ig from animal products are the cost of purification and the need for hyper-immunization to achieve broader specificity (Bostwick et al., 2000). Ig may be derived from a number of commercial sources, including colostrum and milk from different livestock, eggs, and cell culture; however, the most practical sources are bovine milk and colostrum followed by avian eggs (Bostwick et al., 2000).

MOLECULAR PROPERTIES

Occurrence

Ig may be characterized according to their existence in the host, either as serum fractions or as secretory Ig, and because this chapter discusses antimicrobials in food originating from animal sources, the focus will be on secretory Ig from lacteal secretions (milk and colostrum) and avian eggs. There are five general classes of Ig, namely IgG, IgA, IgM, IgE, and IgD. Another specific class, IgY, is exclusively found in eggs (Bostwick et al., 2000). The Ig found in bovine and colostrum whey include (1) IgA; (2) IgG with two major subclasses, IgG₁ (comprising approximately 75% of IgG in milk), IgG₂, and IgG fragments; (3) IgM; (4) J-chain or component; and (5) the free secretory component (Butler, 1994). The nomenclature of Ig is based on their immunologic cross-reaction with reference proteins of human origin and was proposed by the World Health Organization (Butler, 1983; Butler et al., 1971). The protein content of bovine colostrum and milk is higher than that from humans, with Ig levels in bovine colostrum higher than that of humans and, conversely, human milk with higher Ig levels than found in bovine milk (Butler, 1994; Hanson et al., 1993; Kulkarni and Pimpale, 1989). IgG accounts for the majority of the Ig fraction in colostrum and milk (*ca.* 75% to 80%), followed by IgM and IgA at similar levels (Butler, 1994; Hanson et al., 1993; Kulkarni and Pimpale, 1989).

Structure

All Ig molecules are symmetric glycoproteins of approximately 180 kD that are monomers or polymers of four polypeptide chains comprising two identical nonglycosylated light chains (L) (each approximately 20 kD) and two identical glycosylated (with mannoses and N-acetylglucosamine) heavy chains (H) (each approximately 50 to 70 kD) linked together with disulfide bonds (Butler, 1974; Lascelles, 1979; Vasilov and Ploegh, 1982). The H and L chains of each molecule have a constant (C) and a variable (V) region. The V regions are composed of approximately 100 amino acids near the N-terminal, whereas the C region makes up the remainder of the molecule toward the C-terminal (Butler, 1974; Edelman, 1973; Lascelles, 1979). The variable N-terminal regions of both the H and L chains are considered to be important in the specificity of the Ig to

which it can bind, whereas the complement fixation, membrane transport, species-specific, and class-specific Ag determinants are related to the C region of the H chains (Butler, 1974; Lascelles, 1979). The primary difference between the Ig molecules found in bovine colostrum and milk is in the structure of the heavy chains and especially in the V regions (Bostwick et al., 2000).

Biosynthesis

The genes coding for the H and L chains for the production of an Ig molecule are located on different chromosomes and there are separate genes coding for the V and C regions (Bostwick et al., 2000). The genes for the V and C regions on the same chromosome have discrete coding segments or exons separated by noncoding segments or introns, and the mRNA (messenger RNA) produced from these genes undergoes a series of splices to remove the introns, which moves the V and C regions closer together and results in a functional H or L chain (Tonegawa et al., 1976). After synthesis the H and L chains are glycosylated mainly with mannose oligosaccharide (some linked to N-acetylgalactosamine) at asparagines primarily, as well as serine and threonine (Kemp et al., 1983). Assembly of the H and L chains into a complete molecule occurs when cysteine molecules form disulfide bonds, either the formation and crosslinking of two H chains or the combination of an H and L chain (Buxbaum et al., 1971; Parkhouse, 1971). The Ig molecules are synthesized in a class of lymphocytes known as beta cells (B-cells), which may differentiate into plasma cells when activated and secrete Ig (Goldsby et al., 2000; Bostwick et al., 2000). The process of generating mucosal Ig that is found in bovine colostrum and milk begins with the uptake of Ag by M-cells of the Peyer's patches in the intestine or lamina propria of the bronchi (Goldsby et al., 2000). The Ag is presented to B-cells in lymphoid tissue where it is bound preferentially by membrane-bound IgD on the B-cells, activating the B-cells and causing them to secrete IgM, which later switches to IgG (Goldsby et al., 2000). The primed B-cells may then proliferate and differentiate into plasma cells and be directed via lactation hormones to the mammary gland where they produce mucosal Ig in colostrum and milk (Goldsby et al., 2000; Swain et al., 1999).

Isolation and Purification

Bovine colostrum and milk provide ready sources of Ig and many applications may make use of such raw forms of Ig (Korhonen et al., 1998; Reilly et al., 1997), although there are applications that may require extensive purification and as such the type of application determines the degree of purification (Bostwick et al., 2000). The use of colostrum, however, provides some limitation because there is a lower availability with only a few days of production during lactation and the Ig fraction of colostrum decreases significantly after the first 3 days (Kulkarni and Pimpale, 1989). Although milk may be more available and yield a higher volume of substrate, the relatively low concentration of Ig in milk as compared with colostrum makes isolation more difficult (Kulkarne and Pimpale, 1989; Bostwick et al., 2000). Another difficulty associated with isolation of Ig from milk is that milk is most likely pasteurized and the whey is likely to be heat-treated, which results in the loss of activity of the Ig fraction and the decrease in antimicrobial effectiveness (Chen and Chang, 1998). The basic process of Ig isolation and purification from bovine colostrum and milk whey depends on initial concentration and diafiltration of the whey using ultrafiltration techniques followed by purification steps using ion-exchange chromatography to separate other protein fractions and yield a pure, even enriched (Pearce, 1988), Ig fraction and, finally, affinity chromatography to isolate specific Ig molecules (Cordle et al., 1994; Fukumoto et al., 1994; Kanamaru et al., 1993). Bovine colostrum may be centrifuged to remove the fat fraction resulting in an increase of about 30% of the Ig fraction (Cordle et al., 1994; Fukumoto et al., 1994; Kanamaru et al., 1993). Other methods that may be used to purify whey into IgG may be precipitation chromatography, immobilized metal chelate chromatography, gel filtration chromatography, or ion exchange (Bostwick et al., 2000).

Stability

The stability of Ig molecules during processing is affected by thermal treatment (Dominguez et al., 1997; Li-Chan et al., 1995; Lindstrom et al., 1994), and although Ig is heat-sensitive, a substantial proportion of the Ig activity will survive pasteurization (Chen and Chang, 1998). The use of high temperature/short time (HTST) pasteurization techniques results in only 10% to 30% loss of Ig activity, whereas UHT processing and evaporation destroy almost all the specific immune activity (Kummer et al., 1992; Li-Chan et al., 1995). Rapid inactivation of Ig starts at temperatures exceeding 65°C, and it has been demonstrated that at 81°C most of the antiviral activity is lost (Mainer et al., 1999). Anema (2000) indicated that thermal denaturation of Ig is retarded with increasing levels of milk solid concentration. Specific antimicrobial activity of Ig appears to not be affected by storage temperature, retaining its activity up to 12 months of storage at 4°C, 20°C, and 37°C (Husu et al., 1993).

ANTIMICROBIAL ACTIVITY

Mode of Action

Bovine colostrum and milk may contain many naturally occurring antimicrobial substances including the antibody-complement system and complementary antigen-antibody binding activity (Pakkanen and Aalto, 1997; Regester et al., 1997; Reiter, 1985a). The antibody-complement system is considered to be one of the major antimicrobial activities in colostrum (Mueller et al., 1983). The antibodies or Ig absorbed from colostrum after birth in combination with the complement system have a crucial role in conferring passive immunity to a newborn calf (Butler, 1986; Staak, 1992). The Ig-mediated antimicrobial activity of complement is well established in colostrum where all the complement components are present, whereas in milk the activity is not always demonstrated because not all components are present (Eckblad et al., 1981; Husu et al., 1993; Korhonen et al., 1995; Reiter and Brock, 1975). The complement system consists of more than 20 different proteins involved in an enzymatic cascade that may be activated by Ag-Ig interactions (classical pathway), by certain carbohydrates (lectin pathway), or by surfaces not protected by natural inhibitors (alternative pathway) (Korhonen et al., 2000). Complement may kill microorganisms, clear immune complexes, or induce and enhance antibody responses through lytic functions of the membrane-attack complex generated by the cascading enzymes (Fearon, 1998). The activation of the classical pathway involves binding of the first component of complement to Ag-Ig interactions or directly to microbes, whereas in the absence of Ig, complement activation occurs through the lectin pathway with lectins bound to pathogen surfaces or through the alternative pathway, involving the presence of bacterial cell membrane components such as lipopolysaccharides (Holmskov and Jensenius, 1996; Turner, 1996). Although Gram-positive and some Gram-negative bacteria are resistant to complement, most Gram-negative bacteria are sensitive to the lytic actions of complement and both may become inactivated as a result of opsonization and subsequent opsonophagocytosis (Rautemaa and Meri, 1999).

Aside from complement activation, the major Ig-augmented antimicrobial activity occurs through the highly specific, reversible, noncovalent Ag-Ig interactions responsible for immobilizing target Ag as well as sequestration of other components of the immune system to remove the Ag (Bostwick et al., 2000). The mechanisms that may be used by Ig to target an Ag include agglutination, opsonization, adherence-blockade, toxin and virus neutralization, or stasis and cidal activity. Agglutination is brought about by the flexibility in Ig molecules, allowing cells with common surface Ag to be cross-linked to one another and usually involves flagella and pili and, to a lesser extent, the cell membrane. Opsonization or covering of target Ag by Ig molecules may serve to signal effector immune cells to assist in targeting, enhancing phagocytic activity, and even destroying foreign bodies. Ig may bind to specific cell-surface components on microorganisms and form an adherence blockade, preventing the microbe from binding to its host cell-surface receptor.

Although Ig are rarely cidal, they do tend to display stasis more often by binding to microorganisms and causing alterations or disturbances within the cells by impairing growth, multiplication, and other cellular processes until other immune effectors may aid in killing or removing the Ag. Additionally, Ig may serve to neutralize toxins and viruses by binding to various portions of the cells or molecules and thereby prevent receptor-mediated internalization of the Ag molecules.

Specificity

Ig molecules are one of the most effective antimicrobial agents in their ability to target a wide spectrum of pathogenic agents including bacteria, viruses, fungi, protozoa, toxins, and other proteinaceous or polysaccharide molecules as a result of the polyclonal nature of their composition. The two major effects on pathogenic agents involve blockage of attachment and invasiveness or immobilization followed by destruction or removal of Ag regardless of its nature and origin.

APPLICATIONS IN FOOD

Commercial whey or colostrum Ig have been used for many years as a feed supplement for farm animals, mainly newborn, to combat contagious diseases and have proved useful particularly against diarrheal diseases (Korhonen et al., 2000). More recently, commercial products containing milk Ig have been developed and marketed for human use in the prevention and/or treatment of microbial disease (Ebina, 1992; Reddy et al., 1988). The main sources of Ig for commercial production are bovine milk and colostrum because of their availability and safety as compared with serum-derived analogs (Korhonen et al., 2000). Hyperimmune milk or whey derived from the immunization of cows with specific antibodies has been produced and found to contain higher levels of Ig than that of nonimmunized cows (Casswall et al., 1998; Cordle et al., 1991; Facon et al., 1995; Greenberg and Cello, 1996; Kelly et al., 1996; Tacket et al., 1992) and has indeed been marketed in Asian countries for many years (Casswall et al., 1998, 2000; Hilpert et al., 1987). The extent of the increase in specific activity of Ig appears to be dependent on the specific organism and agent used for vaccination (Li-Chan et al., 1995; Facon et al., 1995; Tomita et al., 1995). Li-Chan et al. (1995) found increased ELISA activity for only one of five bacterial Ag in immunized cows in comparison to the levels of activity in milk from commercial dairies. Tomita et al. (1995) found that although cows vaccinated with a lipopolysaccharide–protein conjugate derived from *E. coli* J5 enhanced serum antibody titer to the organism, it did not enhance whey IgG titers. Several studies have suggested that feeding hyperimmune milk increases Ig against the immunizing bacteria and may reduce disease (Boedeker et al., 1987; Chernokhvostova et al., 1990; Murosaki et al., 1991). In early studies, Stephan et al. (1990) found that Ig preparation from the first colostrum of multiple cows displayed antimicrobial activity against a number of microorganisms and when tested *in situ* in a human host conferred Ig against *Cryptosporidium* (Shield et al., 1993). Ormrod and Miller (1991) reported that Ig from milk of dairy cows immunized with a multivariate bacterial vaccine had antiinflammatory activity in the rat hind-paw edema assay. Kobayashi et al. (1991) and Ishida et al. (1992) showed that hyperimmune milk conferred a higher survival rate for mice given lethal doses of irradiation as compared to control mice not receiving the milk. Although bovine colostrum and milk appear to be the ideal sources of Ig for human use, they originate from a foreign species and, as such, are limited to application only against oral and gastrointestinal pathogens or for topical applications in humans (Korhonen et al., 2000).

OVOGLOBULINS

Introduction

Much like mammalian species that produce Ig in serum and lactations, domestic avian species such as chickens, turkeys, and ducks produce Ig in serum and in their eggs (Rose et al., 1974). The Ig

in the avian serum is transferred to the yolks of the eggs to provide the offspring with acquired immunity to avian diseases and other Ag, leaving the newly hatched chick relatively immunocompetent (Rose and Orleans, 1981). Although avian serum contains three main Ig (IgG, IgM, and IgA), the Ig found in egg yolk is referred to as IgY (Leslie and Clem, 1969) as a result of the different structure and immunologic properties as compared with mammalian IgG (Akerstrom et al., 1985; Jensenius et al., 1981; Higgins, 1975; Kobayashi and Hirai, 1980). In comparison with mammalian IgG, IgY is much larger (Kobayashi and Hirai, 1980), more acidic, and less rigid and it does not fix complement (Higgins, 1975).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

Avian blood contains at least three distinguishable kinds of Ig, including IgG, IgM, and IgA, of which IgG comprises approximately 75% of the total Ig (Leslie and Martin, 1973). IgG (known as IgY in egg yolk) is transferred from the maternal serum to the egg yolk and subsequently to the circulation of the chick through the endoderm of the yolk sac, whereas IgM and IgA are secreted into the ripening egg follicle and incorporated into the egg via the oviduct (Patterson et al., 1962; Locken and Roth, 1983). Subsequently, transfer of Ig to the embryonic gut occurs when amniotic fluid is swallowed and thereafter may provide passive immunity to the newly hatched chick (Losch et al., 1986). Hens lay an average of 240 eggs per annum, and the associated production of IgY totals 24 g, which is relatively high in comparison with certain mammals such as rabbits, mice, and goats (Leslie and Clem, 1969; Hatta et al., 1997). In a study comparing two species of chickens, it was determined that although the ratio of yolk weight to egg white was similar in both types of chickens and that the total content of IgY in the yolk was relatively constant during 18 weeks, the level was influenced by hen species, egg weight, and egg production per day (Li et al., 1998).

Isolation and Purification

Egg yolk may be separated by centrifugation into particles and supernatant or plasma (Stadelman and Cotterill, 1977). The plasma portion accounts for about 78% of the total yolk and is composed of a lipid-free globular protein, livetin (existing in three forms, α -, β -, and γ -livetins or IgY) (MaCully et al., 1962). The livetins are water-soluble proteins and exist together with lipoprotein; as such separation of IgY requires extraction of the water-soluble fraction from lipoprotein and subsequent purification of isolated livetins (Polson and von Wechmar, 1980). Separation of the water-soluble fraction from the water-insoluble fraction may involve water dilution of the egg yolk to achieve low ionic strengths and assist the aggregation of the lipoproteins followed by centrifugation or filtration (Akita and Nakai, 1992; Kwan et al., 1991). Once the water-soluble fraction is collected, IgY is separated from α - and β -livetins and purified by a variety of means including ultracentrifugation, organic solvents, precipitation using polyethyleneglycol, precipitation using sodium dextran sulfate, ultrafiltration, ion-exchange chromatography, and metal chelate interaction chromatography (Sim et al., 2000).

Chemistry and Structure

IgY, much like the Ig, found in serum consists of two heavy (H) chains and two light (L) chains and has a molecular weight of approximately 180 kDa (Parvari et al., 1988). Unlike the H chains of mammalian IgG, which have three constant (C) regions and a hinge region, IgY has four C regions and no hinge (Parvari et al., 1988). The content of β -sheet structure in the constant region of IgY is lower than that of mammalian IgG and the flexibility of the region corresponding to the hinge region in IgG was less than that of IgG (Ohta et al., 1991; Shimizu et al., 1992). The lack of disulfide linkage in the IgY L-chain, lower flexibility in the hinge region, and other structural

properties (molecular size, intramolecular bonding, domain conformation) may all influence the lower molecular stability of IgY as compared with IgG (Shimizu et al., 1992).

Stability

The stability of IgY was compared to that of mammalian IgG when exposed to adverse pH and temperature environments (Hatta et al., 1993; Shimizu et al., 1988, 1992, 1993; Otani et al., 1991). In doing so, it was determined that at temperatures higher than 70°C, IgY was more sensitive to the effects of heat than IgG and the maximum temperature of denaturation for IgY was 73.9°C, as compared with 77°C for IgG (Hatta et al., 1993). Shimizu et al. (1988, 1992, 1993) found that heating for 15 minutes at 65°C or higher, especially at temperatures higher than 75°C, reduced the activity of IgY. Otani et al. (1991) reported that at pH 2 and 3, IgY activity was more sensitive to the acidic conditions than IgG. Similarly, it was found that the activity of IgY was decreased at pH values below 3.5 and almost lost at values of 3.0; at the opposite extreme, alkaline conditions up to pH 11 did not affect activity, whereas incubation at pH 12 and higher resulted in a significant decrease (Shimizu et al., 1988, 1992, 1993). It is well established that although IgY is sensitive to pepsin digestion, it is relatively resistant to digestion by trypsin or chymotrypsin and that overall it is more sensitive to digestion by all three enzymes in comparison to IgG (Hatta et al., 1993; Otani et al., 1991; Shimizu et al., 1993).

ANTIMICROBIAL ACTIVITY

Mode of Action

The essential mechanism for Ig to clear foreign material including pathogenic microbes is related to the specific binding to complementary Ag. The Ag–Ig interaction is often referred to as the “lock-and-key” mechanism for the specific noncovalent interactions including hydrogen bonds, hydrophobic bonds, coulombic interactions, and van der Waals forces (Sim et al., 2000). The target Ag may be composed of amino acids, nucleic acids, carbohydrates, or lipids, and it is the strength of association and complementarity that determines the overall binding strength (Sim et al., 2000). IgY has mostly been documented for its antiviral and antibacterial activity achieved by exclusion of these pathogenic agents from infection of host cells. For infection to develop in a host, the etiologic agent needs to form an association with the host cells either by contact or by subsequent internalization. Viruses express surface receptor molecules specific for the host-cell membrane to initiate and assist in internalization of the infective material; thus, the potential for preventing this is the ability of Ig to specifically bind to the viral receptor and block infection, a process termed viral neutralization (Goldsby et al., 2000). Bacterial pathogens are similar to viruses in that most bacteria need to form an adherence with the host cell before the infection process is initiated and the adhesion to the host cell usually triggers invasive mechanisms of the bacteria or toxin production, which may be internalized to cause infection. It was demonstrated that antifimbriae Ig prevented the attachment of bacteria to piglet intestinal epithelia by blocking the mucosal receptor, interfering with binding to mucins, or neutralizing the colonization factor (Imberechts et al., 1997; Jin et al., 1998; Wanke et al., 1990).

Specificity

IgY has been shown to be specific against infectious pathogens of bacterial or viral origin. Specific IgY activity against pathogenic agents has been elucidated from *in vitro* studies in hens immunized with pathogens and through passive immunization of animals with specific IgY (Sim et al., 2000). Antibacterial effects involving the use of specific IgY *in vivo* or *in vitro* have included use of *Salmonella* species, *E. coli*, *Streptococcus mutans*, *Edwardsiella tarda*, *S. aureus*, and *Pseudomonas*

aeruginosa (Akita et al., 1998; Hamada et al., 1991; Hatta et al., 1994, 1997; Ikemori et al., 1992; Jin et al., 1998; Ozpinar et al., 1996; O'Farrelly et al., 1992; Wiedemann et al., 1991; Yokoyama et al., 1992; Yoshiko et al., 1996). Antiviral effects involving the use of specific IgY *in vivo* or *in vitro* have included use of rotavirus, coronavirus, and infectious bursal disease virus (Ebina, 1996; Eterradossi et al., 1997; Ikemori et al., 1997; Kuroki et al., 1993, 1994, 1997).

APPLICATIONS IN FOOD

IgY may find application as a microstatic agent in preventing growth of pathogenic bacteria using specific polyclonal Ig to effectively reduce or neutralize bacterial proliferation in food products and especially meat, thereby reducing the food safety risk associated with these products (Sim et al., 2000). It was suggested that IgY could be used as an ingredient for foods or even mouthwashes to prevent the colonization of invading microorganisms (Hatta et al., 1997). Furthermore, IgY may be used as a food adjuvant to control bacterial growth and prevent attachment of microorganisms to the intestinal epithelium (Sim et al., 2000). Akita and Nakai (1993) proposed the use of IgY in infants younger than 6 months old because the reduced acidity of infant gastric acid would allow the molecule to maintain its protective effect against peptic digestion. Although numerous studies have documented the role of colostrum and milk in conferring protection to newborns (Jason et al., 1984), recent studies proposed the potential of nonmaternal Ig for passive immunization of the gastrointestinal tract (Hatta et al., 1997; Yokoyama et al., 1992). Passive immunization may be the most valuable application of Ig and involves the introduction of pathogen-specific IgY into a host to combat infectious diseases (Hatta et al., 1997). However, for passive immunization to be implemented, large amounts of Ig would need to be administered to an individual and may be achieved through commercial-scale production from eggs laid by hens (Hatta et al., 1997).

OTHER ANTIMICROBIALS OF ANIMAL ORIGIN

AVIDIN

Introduction

Avidin is a 66-kDa, positively charged glycoprotein isolated from various avian egg whites and egg jelly of invertebrates (Green 1964; Gyorgy et al., 1940). Avidin received much attention as a result of its antinutritive effects (Green, 1975); however, subsequent interest has revolved around its ability to bind up to four biotin molecules and form stable complexes (Green, 1964, 1975). The interaction between avidin and biotin is the strongest known protein–ligand binding found in nature (Boas, 1927). The discovery of an antibacterial molecule, streptavidin, produced by *Streptomyces* species (a 60-kDa avidin analog), yielded a similar primary structure compared to avidin and indeed confirmed early suspicion that avidin possesses antimicrobial properties (Bayer and Wilchek, 1990).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

Avidin is a minor protein in avian albumen accounting for only 0.05% of the egg white (Mine, 2000). Avidin production occurs in the goblet cells of the epithelium of the oviduct exclusively in laying hens, indicating that the production is regulated by functioning of the ovaries and specifically the hormone progesterone and several steroids (Elo and Korpela, 1984; Mine, 2000).

Isolation and Purification

Early isolation methods involved the use of selective solubilization of avidin with dilute salt from alcohol-precipitated egg proteins (Dhyse, 1954; Eakin et al., 1941). Alternatively, egg proteins were adsorbed on bentonite and eluted with a solution of dipotassium phosphate followed by purification with ammonium sulfate fractionation (Fraenkel-Conrat and Fraenkel-Conrat, 1952). Adsorption methods were improved with the use of cellulose ion exchange allowing the adsorption of basic proteins on carboxymethylcellulose at high pH and subsequent elution with ammonium carbonate (Melamed and Green, 1963). More recently, methods have included affinity chromatography using biocytin sepharose or iminobiotin columns (Cautrecasas and Wilchek, 1968; Heney and Orr, 1981) to bind avidin for further elution, whereas Durance and Nakai (1988a,b) reported better recovery using a single-column cation exchange as compared to ion exchange methods.

Chemistry and Structure

Avidin is a basic glycoprotein consisting of four identical subunits, each with an approximate molecular weight of 16 kDa (approximately 66 kDa for the molecule), and has a *pI* of approximately 10 (Green, 1975; Woolley and Longworth, 1942). Each subunit consists of 128 amino acid residues and a single intramolecular disulfide bond between cystine residues at position 4 and 83 (DeLange and Huang, 1971). Each subunit is organized in an eight-stranded antiparallel orthogonal β -barrel with extended loop regions providing the biotin-binding pocket (Livnah et al., 1993; Pugliese et al., 1994). Avidin binds one biotin molecule to each of the four comprising subunits, specifically in the highly complementary polar, protein-core pocket, and in the absence of biotin the site may be partially occupied by a water molecule (Gitlin et al., 1988; Pugliese et al., 1994). The structure of avidin reveals the existence of several surface-exposed lysine and arginine residues, which may contribute to the basic nature of the molecule (Hendrickson et al., 1989). Avidin possesses a carbohydrate moiety (about 10% of the total molecular weight) that is comprised of a single oligosaccharide chain with four to five mannose and three N-acetylglucosamine residues linked to *Asp-17* of each polypeptide subunit (Kett et al., 2003).

Stability

Avidin may be resistant to treatment with iodine applied at neutral pH, acetylation of the amino groups, and esterification of the carboxyl groups; however, inactivation of the molecule may result from oxidation with H_2O_2 in the presence of Fe^{2+} or treatment with formaldehyde in the presence of alanine or hydroxylamine at 50°C (Fraenkel-Conrat and Fraenkel-Conrat, 1952). Although avidin is relatively stable over a wide range of pH and temperature in regards to its biotin-binding ability, all four tryptophans associated with the molecule are rapidly oxidized at pH 4.0 and biotin-binding activity is lost when at least two are destroyed (Green, 1963). Avidin may also lose stability at low ionic strength, with 0.1M HCl, 0.1 M sodium-dodecyl-sulfate, and 6 M guanidine HCl resulting in dissociation of avidin subunits and loss of biotin-binding ability (Green, 1963). In addition, avidin may be denatured at temperatures exceeding 70°C; however, the avidin–biotin complex offers stabilization to the molecule up to 100°C (Gitlin et al., 1988; Pugliese et al., 1994).

ANTIMICROBIAL ACTIVITY

Mode of Action

Although the antimicrobial activity of avidin has not been established, it has been suggested that the compound is involved in antimicrobial responses based on the production of streptavidin by *Streptomyces* in the derivation of an antibiotic system and supported by initiation of avidin production at the site of tissue injury in chickens (Elo and Korpela, 1984). It is proposed (Elo et al.,

1978; Korpela, 1984) that avidin production and its secretion by macrophages is induced during inflammation and cellular damage and as such may constitute a host-defense factor for bacterial and viral infection. Studies (Miller and Tauig, 1964) indicating increased amounts of avidin in chicken tissues after intraperitoneal and intravenous administration of *E. coli* support the view that avidin is directed toward combating microbial infection. Korpela (1984) revealed that bacterial binding of avidin was independent of the saturation of its biotin-binding site and in *E. coli* the receptor was the porin protein of the outer membrane. Porins extend through the outer membrane of Gram-negative bacteria and are indeed among the most abundant proteins of the cell constituting an integral part of the structure and function, and because no porins or similar analogs exist in Gram-positive bacteria it is assumed that porin is the only avidin-binding component of the cell envelope (Lugtenberg and van Alphen, 1983). It has been hypothesized (Campbell et al., 1972; Eakin et al., 1941; Herts, 1946) that as a result of the high affinity avidin has for biotin, it may function as an antimicrobial by rendering it unavailable to biotin-requiring microbes; indeed it has been shown to inhibit the *in vitro* growth of biotin-requiring yeasts and bacteria (Green, 1975). Furthermore, binding of extracellular/intracellular biotin may even render it unavailable to the animal cell, thereby resulting in decreased activity of biotin enzymes and altered cellular metabolism and growth (Messmer and Young, 1977; Miller and Tauig, 1964; Wood and Barden, 1977).

Specificity

Although there is very little documentation of the inhibitory effects of avidin on pathogenic bacteria, Korpela (1984) reported on the *in vitro* binding of avidin to various Gram-negative and Gram-positive bacteria. The Gram-negative bacteria found to bind avidin were *E. coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *P. aeruginosa*, whereas the Gram-positive bacteria that bound avidin were *S. aureus* and *S. epidermis*.

APPLICATIONS IN FOOD

Because little is known about the antimicrobial activity of avidin, there are no current applications of this glycoprotein in food as an antimicrobial. However, avidin is used in the avidin–biotin system as a diagnostic tool in immunoassays, and there is potential for application of avidin as an antimicrobial compound in foods (Mine, 2000).

LACTOLIPIDS

Introduction

Lipids, especially fatty acids, isolated from milk and other dairy products possess both nutritional and protective qualities (Kabara, 1978). The antimicrobial activity of lipids and their esters is covered in detail in Chapter 11. Lipids were identified to possess antimicrobial activity in the mid 19th century when it was evidenced that fatty acids generated during the ripening of cheese prevented neurotoxin production by *C. botulinum* (Grecz et al., 1959). In neonates, the antimicrobial activity conferred by milk lipids is a result of the release of antimicrobial fatty acids and monoglycerides from milk triglycerides present in fat globules, which constitute 98% of milk fat (Hamosh, 1991, 1995; Jensen, 1995). Other lipids of animal origin that have provided evidence of antimicrobial activity include epidermis-derived skin lipids shown to inactivate *S. aureus* (Bibel et al., 1989; Miller et al., 1988), free fatty acids at mucosal surfaces shown to inactivate pneumococci (Coonrod, 1987), and porcine intestinal lipids shown to inactivate *Clostridium perfringens (welchii)* (Fuller and Moore, 1967). Lipids may serve to inhibit the establishment, multiplication, and proliferation of pathogenic microorganisms in a host, and their effects may be enhanced by interaction with other antimicrobial factors present in milk (i.e., lactoferrin, lactoperoxidase, lactoglobulins, etc.) (Mandel and Ellison, 1985; Watanabe et al., 1984).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

Fatty acid biosynthesis occurs after a meal when the body is energy rich (Drackley, 2000). The events after consumption involve generation of adenosine triphosphate (ATP) by glycolysis, generation of NADPH (the reduced state of nicotinamide adenine dinucleotide phosphate) by the pentose phosphate pathway, and the storage of glucose as glycogen. Any excess glucose is converted to fatty acids and stored as triacylglycerols (Drackley, 2000). The majority of fatty acid biosynthesis occurs in the cytosol of the liver, although there is limited biosynthesis in other tissues, including adipose tissue, the brain, and mammary glands (Drackley, 2000; Jensen, 1996). The antimicrobial activity in milk is contained mainly in long-chain unsaturated fatty acids and the medium-chain saturated fatty acids (Lampe and Isaacs, 2000). The presence of fatty acids in milk originates from the diet, the mobilization of stored fatty acids, and the synthesis in the mammary glands (Jensen, 1996). The diet of lactating animals affects the proportion of fatty acids present in the milk because a high-energy meal will increase production of medium-chain fatty acids and thereby promote the antimicrobial properties of the milk (Francois et al., 1998; Spear et al., 1992).

Isolation and Purification

Fatty acids are rarely found in free form, and in biological systems such as milk they are generally combined in more complex molecules through ester or amide bonds (Baumann, 1972). Specifically, antimicrobial monoglycerides in milk are bound to a glycerol backbone by ester linkages (Baumann, 1972; Isaacs et al., 1994; Lampe et al., 1998). Analysis of common fatty acids is usually performed by gas-liquid chromatography (GLC) or high performance liquid chromatography (HPLC) (Kroumova and Wagner, 1995). Silver ion chromatography, reversed-phase HPLC, and use of columns have previously been described in the separation of polyunsaturated fatty acids (Kramer et al., 2002) and trans fatty acids, including elaidic acid and geometric isomers of oleic and linoleic acids, that may be found in milk and milk products (Adlof and Lamm, 1998; Christie, 1997). Infrared spectroscopy, GLC, thin-layer chromatography, argentation thin-layer chromatography, liquid chromatography, and mass spectrometry have been described in the isolation of trans fatty-acid isomers in milk (Ledoux et al., 2000; Precht et al., 2001). Modification of ester linkages to ether linkages in monoglycerides through biochemical manipulation results in a compound that is more stable and of equal or greater antimicrobial activity (Isaacs et al., 1994; Lampe et al., 1998).

Chemistry and Structure

Fatty acid molecules are best defined by a description of the length of the carbon chain (number of carbon molecules) and the number of double bonds present and their exact positioning (Drackley, 2000). Common fatty acids are straight-chain compounds and usually possess an even number of carbon atoms (Drackley, 2000). Fatty acid chain lengths are classed into three categories based on their length, namely short-chain fatty acids with a chain length between 2 and 4, medium-chain fatty acids with between 6 and 10 molecules, and long-chain fatty-acids commonly with 12 to 24 carbon molecules but potentially up to 80 (Garrett and Grisham, 1999). The simplest fatty acids have no unsaturated linkages and cannot be modified by hydrogenation or halogenation; these are referred to as saturated fatty acids (Garrett and Grisham, 1999). The presence of double bonds between carbon molecules defines the chains as unsaturated and specifically **monounsaturated** if only one double bond is present and **polyunsaturated** if two or more double bonds are present (Garrett and Grisham, 1999). Alternatively, fatty acids in animals may possess branched chains or contain a variety of other functional groups including acetylenic bonds; epoxy-, hydroxy-, or keto-groups; and even ring structures (Garrett and Grisham, 1999). Monoglycerides or monoacylglycerols are fatty acid monoesters of glycerol and are found in very low amounts in cell extracts as

the intermediate product in the degradation of triacylglycerides or diacylglycerides during lipolysis (Garrett and Grisham, 1999). Monoglycerides are the most polar components of simple lipids because they possess only one hydrocarbon chain and two alcohol groups and thus need careful manipulation to prevent their loss in hydrophilic solutions and on chromatographic columns (Ledoux et al., 2000; Precht et al., 2001). Furthermore, monoglycerides possess detergent properties and as such they easily form micelles in water solutions (Garrett and Grisham, 1999).

Stability

The presence of proteins, especially albumin, may reduce the antimicrobial activity of fatty acids through specific and nonspecific binding (Shibasaki and Kato, 1978). The antimicrobial activity of specifically unsaturated fatty acids may also be reduced by the presence of other surface active agents such as cholesterol (Ammon, 1985; Kabara, 1978). Because the antimicrobial activity of short-chain fatty acids is a result of the undissociated form rather than the anionic form, their activity is highly pH dependent, with activity decreasing as the degree of dissociation increases (Kabara, 1978; Lundblad and Seng, 1991). The effect pH has on antimicrobial activity is observed mainly with short- and medium-chain fatty acids where the MIC of short-chain fatty acids increases while that of medium-chain acids decreases with corresponding increases in pH (Kabara, 1978). The activity of long-chain unsaturated fatty acids as well as monoglyceride esters and ethers of short-chain fatty acids appear to be unaffected by pH alterations (Kabara, 1978).

ANTIMICROBIAL ACTIVITY

Mode of Action

The antimicrobial products produced by the hydrolysis of milk triglycerides are fatty acids and monoglycerides (Thormar et al., 1987). Milk lipids are mainly antiviral, and it has been shown that short- and long-chain saturated fatty acids have minimal antiviral activity, whereas medium-chain saturated and long-chain unsaturated fatty acids are strongly antiviral (Thormar et al., 1987). Monoglycerides of fatty acids produced by hydrolysis of milk lipids are also effective at inactivating enveloped viruses at 5 to 10 times lower concentrations than their corresponding fatty acids (Thormar et al., 1987). In addition, the antiviral activity of fatty acids and monoglycerides is additive (Isaacs and Thormar, 1990). Isaacs and Thormar (1990) demonstrated that the virus envelope is the target for lipid-dependent viral inactivation. Furthermore, research (Noseda et al., 1989; Verdonck and van Heugten, 1997) indicates that antimicrobial lipids destabilize the membrane of cells. The antimicrobial activity of lipids through membrane destabilization has also been demonstrated against fungi and bacteria (Kabara, 1978; Shibasaki and Kato, 1978). The mechanisms by which lipids target microorganisms may be through disruption of the bacterial cell wall or membrane or envelope in viruses, blockage of receptor-ligand interactions, inhibition of intracellular replication, or inhibition of an intracellular target (Lampe and Isaacs, 2000).

Specificity

The antimicrobial activity of milk lipids has been demonstrated on a variety of enveloped viruses including herpes simplex virus, influenza virus, respiratory syncytial virus, measles virus, vesicular stomatitis virus, visna virus, mouse mammary tumor virus, dengue virus types 1 to 4, cytomegalovirus, Semliki forest virus, Japanese B encephalitis virus, and human immunodeficiency virus (Lampe and Isaacs, 2000). Milk lipids have also been shown to inactivate Gram-positive bacteria including *S. epidermis*, *S. aureus*, *C. botulinum*, *B. subtilis*, *B. cereus*, *Streptococcus* species, *Micrococcus* species, *Pneumococcus* species, *Corynebacterium* species, and *L. monocytogenes*; they also have inactivated Gram-negative bacteria including *P. aeruginosa*, *E. coli*, *S. Enteritidis*, *C. trachomatis*, and *N. gonorrhoeae* (Hernell et al., 1986; Isaacs et al., 1990; Kato and Shibasaki,

1975; Lampe et al., 1998; Qu et al., 1996; Reiner et al., 1986; Rohrer et al., 1986; Rabe et al., 1997; Wang and Johnson, 1992, 1997; Wang et al., 1993). In addition, milk lipids display antimicrobial activity against fungi including *Aspergillus niger* and *Trichoderma viride*; yeasts including *S. cerevisiae*, *C. albicans*, *C. utilis*, *Cladosporium species*, *Alternaria species*, and *Kluveromyces marxianus*; and the protozoal pathogen *G. lamblia* (Isaacs et al., 1990; Kato and Shibasaki, 1975; Marshall and Bullerman, 1986; Sofos et al., 1998).

APPLICATIONS IN FOOD

The antimicrobial activity of lipids has been used in food preservation for decades (Grecz et al., 1959; Shibasaki and Kato, 1978). The majority of the lipids isolated from animal products such as milk are considered as GRAS chemicals and can as such find application as antimicrobials in food products (Lampe and Isaacs, 2000). Monoacylglycerols have increased the shelf life of various foods including soy sauce, miso, sausage, Worcestershire sauce, sponge cake, and noodles (Shibasaki, 1982; Sofos et al., 1998). In addition to these foods, the lauric acid ester of monoacylglycerol has shown antimicrobial potential in seafood salad; Camembert cheese; and various flesh foods including deboned chicken meat, minced fish, refrigerated beef roasts, and turkey frankfurter slurries (Baker et al., 1985; Hall and Mauer, 1986; Unda et al., 1991; Wang and Johnson, 1997). Monoacylglycerols may also be combined with other antimicrobials (i.e., nisin) to increase their effectiveness in foods (Sofos et al., 1998). Considering that monoacylglycerols lower the heat resistance of certain bacteria and fungi, they may find application in decreasing the heat-treatment requirements of some foods (Kimsey et al., 1981; Sofos et al., 1998). A proposed application of antimicrobial lipids has been the use in infant formulas to provide protection after hydrolysis of the triglycerides into antimicrobial fatty acids and monoglycerides in the gastrointestinal tract following consumption (Isaacs et al., 1995).

DEFENSINS

Introduction

Defensins are a group of antimicrobial peptides folded in a characteristic β -sheet and a framework of six disulfide-linked cysteines (Ganz, 2003; Selsted et al., 1985). Defensins are widely distributed in nature and indeed in mammalian epithelial cells and leukocytes, often at high concentrations, and have a broad spectrum of antimicrobial activity (Ganz, 2003; Selsted et al., 1985).

MOLECULAR PROPERTIES

Occurrence and Biosynthesis

Defensin peptides have been found in all mammals examined, in chickens and in turkeys, and are abundant in cells and tissues active in host defense against microorganisms (Brockus et al., 1998; Harwig et al., 1994; Zhao et al., 2001). The highest (>10 mg/ml) concentration of defensins is usually in the granules or storage organelles of leukocytes (Ganz, 2003). On ingestion of microorganisms by leukocytes into phagocytic vacuoles, the vacuoles fuse with the granules and the granule material is delivered onto the target organism (Ganz, 2003). Another site containing high (>10 mg/ml) concentrations of defensins are the Paneth cells, specialized cells of the small intestine that contain secretory granules that are released into intestinal pits or crypts (Ganz, 2003; Harder et al., 1997). Other cells containing defensins at lower concentrations (10 to 100 μ g/ml) include barrier and secretory epithelial cells, which may produce defensins constitutively or only on infection, and to a lesser extent cells of the immune system such as monocytes, macrophages, and lymphocytes (Agerberth et al., 2000; Harder et al., 1997; Ryan et al., 1998).

Chemistry and Structure

There are two main defensin subfamilies, α - and β -defensins, that differ in peptide segment length between the six cysteine residues and the cysteine pairs connected by disulfide bonds (Hill et al., 1991; Hoover et al., 2000; Sawai et al., 2001; Zhang et al., 1992; Zimmermann et al., 1995). Defensins exist as a triple strand β -sheet that has the distinctive fold brought about by the disulfide bonds between cysteine residues (Hoover et al., 2000; Sawai et al., 2001). The amino acid sequence and composition of defensins are highly variable; however, the cysteine framework is conserved in each defensin subfamily (Ganz, 2003). Most α - and β -defensins possess clusters of positively charged amino acids, although their distribution in the molecule is variable (Ganz, 2003). Subcellular storage organelles such as leukocytes and Paneth cells are rich in negatively charged glycosaminoglycans (Parmley et al., 1986). The majority of α - and β -defensins from leukocytes and Paneth cells contain arginine as the main cationic amino acid, whereas β -defensins secreted from epithelial cells contain similar amounts of arginine and lysine (Fromm et al., 1995; Kostoulas et al., 1997). The general synthesis of α -defensins occurs through the encoding of tripartite prepropeptide sequences to produce an amino acid precursor consisting of 90 to 100 amino acids with an amino (N)-terminal signal sequence (approximately 19 amino acids), an anionic propeptide (approximately 45 amino acids), and a carboxy (C)-terminal mature cationic defensin (approximately 30 amino acids) (Ganz, 2003). The synthesis of β -defensin precursors is similar, consisting of a signal sequence at the N-terminus, a short or absent propeptide, and the mature defensin peptide at the C-terminus; however, it lacks the anionic propeptide that is prominent in the α -defensin precursor (Ganz, 2003).

ANTIMICROBIAL ACTIVITY

Mode of Action and Specificity

Defensins are mainly antibacterial and antimycotic, especially at low ionic strength conditions, low concentrations of divalent cations, plasma proteins, and other interfering substances (Ganz, 2003; Lehrer and Ganz, 2002; Selsted et al., 1993). Under these optimal conditions, defensins may be active at very low (1 to 10 $\mu\text{g/ml}$) concentrations (Ganz, 2003). Defensins have also been shown to be effective against some enveloped viruses (Daher et al., 1986). The main mechanism responsible for antimicrobial activity is attributed to the permeabilization of target membranes and subsequent cell leakage; inhibition of RNA, DNA, and protein synthesis; and decreased cellular viability (Ganz, 2003).

APPLICATIONS IN FOOD

Although defensins are antimicrobial peptides that may be isolated and purified from animals, there is, other than the antimicrobial activities as a natural ingredient in raw food products, no known application of the peptides as an additive in foods.

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16 Sanitizers: Halogens, Surface-Active Agents, and Peroxides

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CONTENTS

Halogens.....	509
Chlorine and Chlorine Compounds.....	509
Mechanism of Action.....	511
Antimicrobial Activity.....	512
Factors Affecting Activity.....	516
Effect of pH.....	516
Effect of Temperature.....	519
Effect of Organic Material.....	519
Effect of Water Hardness.....	521
Toxicity.....	521
Iodophors.....	522
Mechanism of Action.....	522
Antimicrobial Activity.....	523
Factors Affecting Activity.....	524
Effect of pH.....	524
Effect of Temperature.....	527
Effect of Organic Matter.....	528
Effect of Water Hardness.....	528
Toxicity.....	528
Surface-Active Agents.....	528
Quaternary Ammonium Compounds.....	528
Mechanism of Action.....	529
Antimicrobial Activity.....	529
Factors Affecting Activity.....	531
Effect of pH.....	531
Effect of Temperature.....	532
Effect of Organic Matter.....	532
Effect of Water Hardness.....	532
Toxicity.....	532
Acid-Anionic Surfactants and Fatty Acids.....	533
Mechanism of Action.....	534
Antimicrobial Activity.....	534
Factors Affecting Activity.....	534

Toxicity	536
Amphoteric Surfactants.....	536
Mechanism of Action.....	536
Antimicrobial Activity	536
Factors Affecting Activity.....	536
Toxicity	537
Peroxides	537
Hydrogen Peroxide	538
Mechanism of Action.....	538
Antimicrobial Activity	539
Factors Affecting Activity.....	539
Effect of Concentration	539
Effect of pH.....	540
Effect of Temperature.....	540
Effect of Other Factors.....	541
Toxicity	542
Peroxyacetic Acid	542
Mechanism of Action.....	543
Antimicrobial Activity	543
Factors Affecting Activity.....	545
Effect of Concentration	545
Effect of pH.....	547
Effect of Temperature.....	547
Effect of Other Factors.....	549
Toxicity	550
Summary	551
Regulatory Requirements.....	551
Properties for Approved Sanitizers.....	552
Antimicrobial Treatments Applied to Food Tissue Surfaces	552
Red Meats and Poultry	552
Red Meats	553
Chlorinated Compounds.....	553
Organic Acids.....	553
Hydrogen Peroxide.....	553
Peroxyacids.....	555
Trisodium Phosphate	556
Ozone.....	556
Poultry	556
Chlorinated Compounds.....	556
Organic Acids.....	557
Hydrogen Peroxide.....	557
Peroxyacids.....	558
Trisodium Phosphate	558
Ozone.....	559
Cetylpyridinium Chloride	559
Raw Fruits and Vegetables.....	559
Chlorinated Compounds.....	560
Hydrogen Peroxide.....	561
Peroxyacids.....	561
Ozone.....	561
References	562

The chemical sanitizers currently used in the food industry can enter the food chain via several different routes. They occur in finished foods at very low concentrations and are present as indirect or incidental food additives. The routes of entry include the following:

1. Use of sanitizing solutions on food manufacturing equipment surfaces
2. Use of sanitizing solutions on fresh meat and poultry carcasses
3. Use of sanitizing solutions on food service equipment surfaces and as chemical sanitizers on food utensils
4. Use of sanitizers in nonfood contact surfaces in food manufacturing plants
5. Use of sanitizing solutions as teat dips or udder washes on the dairy farm

Guidelines for the use of sanitizing solutions and a listing of approved sanitizing solutions have been reviewed in several publications (Elliot, 1980; Harper, 1980). Much information is included in the summary. This chapter is intended to provide information relating to the chemistry, mechanism of antimicrobial activity, and influence of environmental factors on the efficiency of the various chemical sanitizers. Information relating to the toxicology of these compounds is also discussed.

In the United States, the Food and Drug Administration (FDA) had joint jurisdiction with the Environmental Protection Agency (EPA) for no-rinse sanitizers until 1996 at which time sole jurisdiction was transferred to the EPA. During that time period FDA approved more than 40 compositions for food contact surfaces.

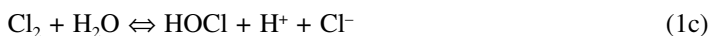
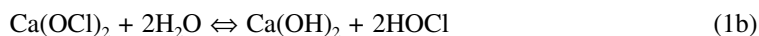
HALOGENS

CHLORINE AND CHLORINE COMPOUNDS

Chlorine in its various forms is one of the most widely used chemical sanitizers in the food industry. Chlorine compounds are used (1) as adjuncts to water used for conveying raw food products, as well as water used for cooling of heat-sterilized cans; (2) as sanitizing solution for food contact surfaces; and (3) in the treatment of raw meat, poultry, fish, and produce to reduce microbial load and to extend shelf life.

Several types of chlorine compounds are available (Table 16.1), with the hypochlorites the most commonly used chlorine compounds in food manufacturing and food service. Chlorine-based sanitizers form hypochlorous acid (HOCl) in solution. "Available" chlorine may be defined as a measurement of oxidizing capacity and is expressed in terms of the equivalent amount of elemental chlorine (Dychdala, 2001). In general, the organochlorines are slower-acting bactericides than the inorganic forms, but they offer the advantage of stability and are relatively less irritating to personnel and less corrosive to equipment.

The chemistry of chlorine in solution, whether the source is elemental chlorine, hypochlorites, or organochlorines, can basically be described as follows:



The term "free available chlorine" is usually applied to the three forms of chlorine that may be present in water. These forms are (1) elemental chlorine (Cl_2), (2) HOCl, and (3) hypochlorite ion (OCl^-). At pH 4 to 5, a majority of the chlorine is in the HOCl form. As the pH is decreased below 4.0, increasing amounts of Cl_2 are formed; above pH 5.0, OCl^- proportions increase.

TABLE 16.1
Typical Characteristics of Chlorine and Chlorinated Compounds

Name	Chemical Formula	Chemical Abstracts Registry No.	Approximate Maximum Water Solubility at 25°C (%)	Commercial Form	Typical Commercial Available Chlorine%
Gaseous chlorine	Cl ₂	7782-50-5	0.76 ^a	Pressurized liquid	100 ^b
Hypochlorous acid	HOCl	7790-92-3	60	Aqueous solution (typically prepared on site)	Varies up to 41
Sodium hypochlorite	NaOCl	56802-99-4	45	Aqueous solution	5-15
Calcium hypochlorite	Ca(OCl)·(H ₂ O) _x	7778-54-3	21	Powder	32-65
Potassium hypochlorite	KOCl	7778-66-7	60	Aqueous solution	5-15
Lithium hypochlorite	LiOCl	13840-33-0	40	Powder	35
Chlorinated trisodium phosphate	(Na ₃ PO ₄ ·11H ₂ O) ₄ ·NaOCl	56802-99-4	18	Powder	3.65
Chloramine-T	C ₇ H ₇ ClNNaO ₂ S	473-34-7	15	Powder	23-26
Dichlorodimethyl-hydantoin	C ₅ H ₆ Cl ₂ N ₂ O ₂	4118-52-5	0.2	Powder	66-70
Trichloro(iso)cyanuric acid	Cl ₃ (NCO) ₃	87-90-1	1.2	Powder	90
Sodium dichloro(iso)cyanurate	NaCl ₂ (NCO) ₃	2893-78-9	25	Powder	62 (55 dihydrate)
Chlorine dioxide	ClO ₂	11049-04-4	1	Aqueous gas (prepared on site)	c

^a As total of (Cl₂ + HOCl + Cl) moieties.

^b By definition.

^c Chlorine dioxide does not form HOCl in solution and has “available chlorine.” Oxidizing potential can be given as “equivalents” of available or as active chlorine. One gram of ClO₂ is equivalent to 2.63 g of active chlorine.

Source: Adapted from Cords et al. (2001).

TABLE 16.2
Relation between Hypochlorous Acid (HOCl)
Content and pH

pH	Amount of Chlorine Present as HOCl Species (%)
4.5	100
5.0	98
7.0	94
7.0	75
8.0	23
9.0	4
10.0	0

Source: Adapted from Baker (1959).

Table 16.2 illustrates the relative percentages of the HOCl species present over a wide pH range. Hypochlorous acid is the most bactericidal species of the three; however, the other forms possess some antimicrobial activity.

In recent years chlorine dioxide has gained greater prominence in disinfection. The chemistry of chlorine dioxide (ClO_2) differs from that of the other chlorine compounds in that hypochlorous acid is not formed from reduction of ClO_2 . It is used for drinking water disinfection, for in-plant chlorination, for wastewater treatment, for cooling water tower maintenance, and for the bleaching of paper. Compared to chlorine, chlorine dioxide has a number of unique properties: (1) the ability to break down phenolic compounds and remove phenolic tastes and odors from water; (2) the inability to form trihalomethanes; (3) the capability to eliminate cyanides, sulfides, and mercaptans from wastewater; and (4) nonreactivity with ammonia. Chlorine dioxide exhibits a number of similarities with chlorine in its oxidation-reduction potential, in tests to assay the solution concentration, and in antimicrobial activity (Knapp and Battisti, 2001).

More recently, the use of acidified sodium chlorite (ASC), a combination of an organic acid and sodium chlorite, has shown promise as an effective sanitizer in the food processing environment. ASC chemistry is principally that of chlorous acid (HClO_2), which forms on acidification of chlorite (Warf and Kemp, 2001). Working solutions, typically with an operational pH of 2.3 to 3.2, must be prepared on site from the dissociation of chlorite by a weak acid.

MECHANISM OF ACTION

Dychdala (2001) reviewed the mechanisms of bactericidal activity for chlorine. Several theories have been advanced, including cell membrane effects, inhibition of sulfhydryl enzymes, and inhibition enzymes involved in glucose metabolism. In a paper by Haas and Engelbrecht (1980), the authors concluded that chlorine is capable of producing lethal reactions at or near the cell membrane, as well as affecting DNA. It has also been suggested the NaOCl reacts with the DNA of living cells, causing mutation by oxidation of purine and pyrimidine bases (Rosenkranz, 1973; Wlodkowski and Rosenkranz, 1975). In an investigation to determine the mechanism of inactivation of *Escherichia coli* by monochloramine, it was concluded that the disinfectant did not severely damage the cell envelope of the bacterium or affect nucleic acid function. Rather, cell death was associated with typical protein-associated activities including membrane transport, respiration, and substrate dehydrogenation (Jacangelo et al., 1991).

The mechanism of bactericidal action of chlorine dioxide was reviewed by Knapp and Battisti (2001). Although conflicting results have been observed between studies that have sought to determine its mode of antimicrobial action, several conclusions can be drawn. Amino acids are

rapidly affected by ClO_2 (Noss et al., 1986). Physiologic alterations of the cell by ClO_2 include the inhibition of protein synthesis and the disruption of the outer membrane (Aieta and Berg, 1986). Bernarde et al. (1967b) also attributed inactivation by ClO_2 to a disruption of protein synthesis.

The sporicidal effects of chlorine have been attributed to the extraction of the spore coat and spore cortex peptidoglycan leading to the rehydration of the spore protoplast, which facilitates diffusion of the agent to its site of action (Bloomfield, 1996). Kulikovsky et al. (1975) attributed spore inactivation to permeability damage resulting in a loss of calcium ion, dipicolinic acid, RNA, and DNA.

Few studies have investigated the virucidal effects of low levels of chlorine. Maillard and Hann (1998), using scanning electron microscopy, observed a wide range of deleterious effects following treatment with sodium hypochlorite of bacteriophage. They concluded that hypochlorite-inactivated bacteriophage had structural alterations to the phage head, tail, and overall structure, which may result in the release of viral nucleic acids from damaged capsids to the surrounding medium.

The ability of bacteria to acquire tolerance to chlorine has been demonstrated. Lisle et al. (1998) observed a phenotype of *E. coli* O157:H7 resistant to 0.5 ppm chlorine following adaptation to starvation conditions. This concentration of chlorine, however, is much lower than what is used in sanitizing solutions. Tolerance to chlorine and several other sanitizers has been demonstrated by microorganisms within biofilm communities (Chen and Stewart, 1996; Chen et al., 1993; Sommer et al., 1999).

Chlorine-induced sublethal injury has also been demonstrated (Scheusner et al., 1971; Dukan et al., 1997). Virulence of waterborne enteropathogens was shown to be influenced by chlorine-induced injury (LeChevallier et al., 1985). Following treatment with up to 1.5 ppm chlorine, *E. coli* and *Salmonella* cells lost their ability to invade HeLa cells. Dukan et al. (1997) described three subpopulations of an original population of 10^8 cfu/ml *E. coli* following exposure to 1 ppm chlorine: a majority of dead (nonrespiring) cells, a few culturable cells (10^2 to 10^4), and about 10^7 viable but nonculturable cells. These authors suggested that recovery in nutrient-limited media of an HOCl-stressed populations is in large part because of the growth of a few culturable cells at the expense of damaged cells.

ANTIMICROBIAL ACTIVITY

Chlorine is considered a broad-spectrum germicide. Hypochlorites have documented antimicrobial activity against nonacid-fast bacteria, acid-fast bacilli, bacterial spores, viruses, fungi, algae, and protozoa (Dychdala, 2001).

Vegetative cells of bacteria are generally more susceptible to chlorine inactivation than bacterial spores. The results generated by several authors are summarized in Tables 16.3 and 16.4. Orth and Mrozek (1989) evaluated the effectiveness of sodium hypochlorite against a number of pathogenic organisms, including *Listeria monocytogenes*, *Campylobacter jejuni*, and *Yersinia enterocolitica*. The results of this work showed that different organisms, while displaying varying resistances to chlorine, were completely destroyed by chlorine under practical use conditions of time, temperature, and concentration. In another study, Lopes (1986) reported that 100 ppm available chlorine as sodium hypochlorite and dichloroisocyanurate was effective for the 99.999% reduction of *L. monocytogenes* and *Salmonella* Typhimurium within 30 seconds. Brackett (1987) demonstrated that chlorine concentrations of less than about 50 ppm showed no antimicrobial effect of *L. monocytogenes* after 20 seconds of exposure. Treatment with 50 ppm chlorine or greater, however, resulted in the failure to recover viable cells.

Several authors (Johns, 1934, 1948; Trueman, 1971; Mosley et al., 1976) have observed a greater resistance in certain strains of *Staphylococcus aureus* compared to pseudomonads and other Gram-negative bacteria. With other commonly used sanitizers and disinfectants, notably quaternary ammonium compounds, the reverse is generally true. Haas and Engelbrecht (1980) suggested that yeasts and acid-fast bacteria would be more appropriate indicators of water disinfection than *E. coli*

TABLE 16.3
Inactivation of Bacteria by Hypochlorite

Organism	FAC ^a (ppm)	pH	Temperature (0°C)	Exposure Time	Inactivation (%)	Reference
<i>Aerobacter aerogenes</i>	0.01	7.0	20	5 min.	99.8	Ridenour and Ingols (1947)
<i>Staphylococcus aureus</i>	0.07	7.0	20	5 min.	99.8	Ridenour and Ingols (1947)
<i>Escherichia coli</i>	0.01	7.0	20	5 min.	99.9	Ridenour and Ingols (1947)
<i>Salmonella dysenteridae</i>	0.02	7.0	20	5 min.	99.9	Ridenour and Ingols (1947)
<i>Salmonella paratyphi B</i>	0.02	7.0	20	5 min.	99.9	Ridenour and Ingols (1947)
<i>Salmonella Derby</i>	12.5	7.2	25	15 s	>99.999	Mosley et al. (1976)
<i>Escherichia coli</i>	12.5	7.7	25	15 s	>99.999	Mosley et al. (1976)
<i>Escherichia coli</i>	6	8.6	25	15 s	>99.999	Hays et al. (1967)
<i>Streptococcus lactis</i>	6	8.4	25	15 s	>99.99	Hays et al. (1967)
<i>Lactobacillus plantarum</i>	6	8.4	25	300 s	0	Hays et al. (1967)
<i>Lactobacillus plantarum</i>	6	5.0	25	15 s	>99.99	Hays et al. (1967)

^a Free available chlorine.

because of their greater resistance to free available chlorine. A comprehensive report published by the EPA (1979) ranked *Mycobacterium fortuitum* and *Candida parapsilosis* well ahead of *E. coli* in resistance to chlorine. Whereas concentrations from 0.5 to 5 ppm free chlorine will produce kill of a range of vegetative bacteria in suspension tests within 5 seconds to 5 minutes, a concentration of 50 ppm chlorine was required to kill *Mycobacterium tuberculosis* following 30 seconds of exposure (Dychdala, 2001).

The difference in resistance to chlorine between vegetative cells and bacterial spores has been discussed (Ito and Seeger, 1980; Odlaug, 1981). Based on free available chlorine concentration and contact time, bacterial spores are 10 to 10,000 times more resistant to destruction by chlorine than vegetative cells. Moreover, spores of aerobic bacteria are generally more resistant than spores of anaerobic bacteria. Recent studies have shown that hypochlorites are among the most potent sporicidal agents. Orr and Beuchat (1999) determined that a concentration of 1000 ppm free chlorine as sodium hypochlorite was required to achieve complete inactivation of *Alicyclobacillus acidoterrestris* spores following 10 minutes of exposure, whereas surviving populations were recovered following treatment with 1200 ppm acidified sodium chlorite, 12% trisodium phosphate, 2% hydrogen peroxide, and 160 ppm peroxyacetic acid (PAA). Sagripanti and Bonifacino (1996) demonstrated that hypochlorite and PAA ranked the highest in terms of sporicidal activity when compared to five other antimicrobial agents.

The tenacity of bacterial cells within biofilms to resist inactivation or death by exposure to chlorine and other antimicrobial agents has been repeatedly demonstrated (Carpentier and Cerf, 1993; Anwar et al., 1990; Ronner and Wong, 1993). Resistance is likely the result of the impedance by the biofilm extracellular matrix of chlorine penetration by a reaction–diffusion interaction (Chen

TABLE 16.4
Inactivation of Spore-Forming Bacteria by Hypochlorite

Organism	FAC ^a (ppm)	pH	Temperature (0°C)	Exposure Time (min)	Inactivation (%)	Reference
<i>Bacillus cereus</i>	100	8.0	21	5	99	Cousins and Allan (1967)
<i>Bacillus subtilis</i>	100	8.0	21	60	99	Cousins and Allan (1967)
<i>Bacillus macerans</i>	7.5	6.5	25	8	99.99	Seeger (1978)
<i>Bacillus stearothermophilus</i>	200	6.5	25	9	99.99	Seeger (1978)
<i>Bacillus coagulans</i>	5	6.8	20	27	90	Labree et al. (1960)
PA 3679	6.5	6.5	25	7	99.9	Seeger (1978)
<i>Clostridium botulinum</i> type A	4.5	6.5	25	10.5	99.99	Ito and Seeger (1980)
<i>Clostridium botulinum</i> type E	4.5	6.5	25	6.0	99.99	Ito and Seeger (1980)
<i>Clostridium perfringens</i> 6719	5	8.3	10	60	None	Dye and Mead (1972)
<i>Clostridium histolyticum</i>	5	8.3	10	10	90	Dye and Mead (1972)
<i>Clostridium tertium</i>	5	8.3	10	20	99.9	Dye and Mead (1972)
<i>Clostridium bifermentans</i>	5	8.3	10	20	99.9	Dye and Mead (1972)
<i>Clostridium sporogenes</i>	5	8.3	10	35	99.9	Dye and Mead (1972)

^a FAC, free available chlorine.

and Stewart, 1996). Bolton et al. (1988) showed that strains of *S. aureus* isolated from biofilms and collected from the defeathering equipment were almost eight times more resistant than the strains taken from the natural skin flora of poultry. The authors of this work concluded that the resistance of these strains was the result of their ability to form macroclumps by production of an extracellular slime. Ronner and Wong (1993) demonstrated that recoverable cells within biofilm communities associated with buna-N rubber gasket material were reduced by less than 1 to 2 log₁₀ following treatment with 100 ppm free chlorine. Their planktonic counterparts were completely killed following similar treatment in suspension. Caldwell (1990), working with biofilms, reported that low chlorine levels (0.5 to 5 ppm) are only inhibitory to biofilms and their cells. According to the author, higher chlorine levels, 50 ppm and up, were needed for the reduction of biofilms by biocidal action.

Chlorine compounds have demonstrated cidal activity against bacteriophage. Sing et al. (1964a, b) compared the destructive activity of several germicidal aerosols against *Streptococcus cremoris* phage 144F. These authors found levels of 500 to 2000 ppm available chlorine to be highly effective against bacteriophage on a variety of surfaces. Maillard et al. (1998) studied the effect of sodium hypochlorite on *Pseudomonas aeruginosa* PAO1 phage F116 and found it to be highly sensitive to low concentrations. Reductions of at least 4 log₁₀ were demonstrated following treatment with 75 ppm chlorine for 30 seconds. These authors found that different types of enteric viruses vary widely

in the degree of resistance to free chlorine and that poliovirus, coxsackievirus, and some echoviruses were more resistant than coliform or enteric pathogenic bacteria. Finichiu et al. (1986) studied the resistance of different microorganisms to chlorine to assure good drinking water quality. The authors concluded that the resistance of bacteria to chlorine was much lower than that of viruses.

Berg et al. (1989) reported rapid inactivation by free chlorine of poliovirus 1°C at 5°C at pH 9 in drinking water. It was suggested that different ions from many salts present in the water may potentiate the virucidal activity of chlorine. In a follow-up study, the same authors determined that poliovirus 1 was inactivated approximately 15 times more rapidly by chlorine in pure water containing potassium chloride than in the absence of the salt. These investigations suggest that chlorine levels may be reduced without sacrificing any of the virucidal activity, which may in turn reduce the quantity of trihalomethane formation resulting from chlorination of hydrocarbons.

The fungicidal activity of chlorine has not been as extensively investigated as its bactericidal or sporicidal activity. Cheng and Levin (1970) studied the inactivation of *Aspergillus niger* conidiospores in the presence of 1 to 20 ppm chlorine. A comparison of their findings with those of other authors (Hays et al., 1967; Ito and Seeger, 1980) indicates that fungal spores are slightly more resistant than vegetative bacteria. The authors attributed this difference to the fact that the germicide must travel farther to penetrate the coat of a fungal spore compared to a bacterial cell. Ver Kuilen and Marth (1980) investigated the sporicidal effect of hypochlorite on *Aspergillus parasiticus*. Following treatment with 3 ppm chlorine for 15 minutes, the number of recovered conidia fell by 3.5 log₁₀.

Brown and Wardowski (1986) reported the use of chlorine in citrus packaging housed for the reduction of decay on citrus fruits caused by *Penicillium digitatum* and *Geotrichum candidum*. The use of chlorine or chlorine dioxide under different plant conditions was presented, and effective control of these organisms was reported.

Coccidian intestinal parasites of importance in water disinfection have been demonstrated to be resistant to chlorine at concentrations typically applied for water treatment (Chauret et al., 2001). Venczel et al. (1997) determined that the infectivity of *Cryptosporidium parvum* oocysts was not influenced following treatment with 5 ppm chlorine for 24 hours. Similarly, Korich et al. (1990a,b) concluded that the use of disinfectants alone should not be expected to inactivate *C. parvum* oocysts in water after they observed minor reductions in infectivity following chemical treatment. Research has demonstrated the *C. parvum* oocysts are several times more resistant to chemical sanitizers than cysts of *Giardia lamblia*.

The antimicrobial action of the commercially available organic compounds is generally slower than that of hypochlorite. This difference in rate of kill should be taken into account when rapid rates of kill are essential or desirable. Dychdala (2001) provided a general review of the available organic chlorine compounds. A comparison of several of these compounds with a standard hypochlorite solution against *Salmonella Typhi* is shown in Table 16.5. These data indicate that the type of organic carrier has a definite effect on the germicidal activity of these compounds. Bloomfield and Miles (1979a, b) compared the antibacterial properties of sodium dichloroisocyanurate (NaDCC) and sodium hypochlorite (NaOCl) formulations. They concluded that the NaDCC formulation had a higher antimicrobial activity than NaOCl and that the difference in the activities may be the residual isocyanuric acid and hypochlorous acid present in solution. Odlaug's (1981) review of the literature indicated that higher concentrations and/or longer contact times are required for chloramine to achieve the same degree of kill as NaOCl.

The sporicidal activity of some of the organic chlorine compounds was reviewed by Trueman (1971). For trichloroisocyanuric acid, the sporicidal activity was significantly lower than hypochlorite. However, comparable, if not superior, results were obtained with trichloroisocyanuric acid against sensitive vegetative bacteria compared to hypochlorite. Fundamental differences in the properties and mode of actions exist between sodium hypochlorite and organic chlorine compounds. Chloramine and sodium dichloroisocyanurate have shown superior bactericidal, yet inferior sporicidal, activity (Odlaug, 1981; Coates, 1996).

TABLE 16.5
Effect of pH on Germicidal Activity^a of Organic Chlorine Compounds

Compound	pH				
	6	7	8	9	10
Trichloroisocyanurate	125 ^b	160	175	200	225
Dichloroisocyanurate	125	200	200	200	250
Dichlorodimethylhydantoin	60	150–200	400	2000	3000–4000
Chloramine-T		150–200	500–1000	3000	3000–4000

^a AOAC available chlorine germicidal equivalent test (1980) using *Salmonella* Typhi.

^b Values given are ppm available chlorine required to provide germicidal activity equivalent to 200 ppm available chlorine from sodium hypochlorite at pH 8.5.

Source: Adapted from Ortenzio and Stuart (1959).

Dichloroisocyanuric acid was found to be comparable to sodium hypochlorite in the destruction of bacteriophage (Sing et al., 1964a b). Trichloroisocyanuric acid has also been shown to inactivate bacteriophage at fairly low levels (10 ppm) in aqueous solution (Fortney, 1958). Shin and Sobsey (1998) demonstrated that although Norwalk virus was more susceptible to treatment with monochloramine than poliovirus 1 or coliphage MS2, enteric viruses may not be rapidly or extensively inactivated by disinfection with low concentrations of monochloramine.

Knapp and Battisti (2001) reviewed the literature regarding the antimicrobial efficacy of ClO₂. They concluded that conditions of concentration, pH, and time were quite similar to those reported for chlorine against vegetative cells. They also concluded that higher concentrations were necessary for sporicidal activity, except that chlorine dioxide appeared to be more effective against aerobic than anaerobic sporeformers. Chlorine was found to be generally more effective against anaerobic sporeformers. The higher sporicidal activity of chlorine dioxide compared to chlorine may be attributable to its greater oxidation capacity through the changes of five electrons in the reduction process to chloride ion. A summary of the antimicrobial activity of ClO₂ is depicted on Table 16.6.

Little information is available regarding the efficacy of acidified sodium chlorite in reducing populations of bacteria, viruses, fungi, or protozoa. Miner (2001) reported complete inactivation of *Salmonella* species, *E. coli*, and *E. coli* O157:H7 following exposure to ASC for 30 seconds. Spores of *Bacillus cereus* were completely inactivated by ASC treatment within 5 minutes. Planktonic foodborne pathogens were found to be quite sensitive to ASC.

FACTORS AFFECTING ACTIVITY

The antimicrobial activity of chlorine is highly dependent on environmental factors during exposure of microorganisms to the germicide. Such factors as pH, temperature, organic load, and water hardness can all play significant roles in microbial efficacy.

Effect of pH

The role of pH in antimicrobial efficacy of chlorine is well documented. Dychdala (2001) reviewed much of the earlier research in this area. Table 16.7 clearly illustrates the effect of pH on various vegetative bacteria and yeasts. In this case, the effect on *E. coli* is not readily apparent; however, dramatic effects are evident with *Streptococcus lactis*, *Pediococcus cerevisiae*, and *Saccharomyces cerevisiae*.

TABLE 16.6
Inactivation of Microorganisms by Chlorine Dioxide

Microorganism	ClO ₂ (ppm)	Exposure Time	Temperature (°C)	Percent Inactivation	Reference
<i>Escherichia coli</i>	0.25	40 sec	20	99.000	Ito and Seeger, 1980
<i>Staphylococcus aureus</i>	0.12	5 min	20	99.990	Ito and Seeger, 1980
<i>Bacillus subtilis</i> spores	1.00	5 min	20	99.900	Ito and Seeger, 1980
Aerobic bacteria	5.00	30 min	6	99.970	Reina et al., 1995
Enterobacteriaceae	5.00	30 min	6	>99.999	Reina et al., 1995
Lactic acid bacteria	5.00	30 min	6	>99.900	Reina et al., 1995
<i>Lactobacillus buchneri</i>	6.00	30 min	26	100.000	Han et al., 1999
Yeasts	5.00	30 min	6	>99.700	Reina et al., 1995
Mold	5.00	30 min	6	99.900	Reina et al., 1995
<i>Penicillium expansum</i>	5.00	30 sec	RT	100.000	Roberts and Redmond, 1994
<i>Cryptosporidium parvum</i>	2.50	30 min	13	90.000	Li et al., 2000

For bacterial spores, pH also plays a role in the sporicidal efficacy of hypochlorite. An example of the dramatic effect of pH is the results presented for *Bacillus metiens* (Table 16.8). At pH 10, 121 minutes of exposure was required to obtain the same degree of kill as 2.5 minutes at pH 6 under the same conditions of chlorine concentration and temperature. Most commercial hypochlorite solutions produce a slightly alkaline pH at their use dilution. The antimicrobial properties of chlorine are not as favorable under slightly alkaline conditions; however, the stability of the solution is much improved.

The effect of pH on the antimicrobial activity of N-chloro compounds was reviewed by Trueman (1971) and Russell (1971). Data collected on four of the more commonly used organic chlorine compounds are summarized in Table 16.5. The cyanuric acid derivatives are notably less affected by pH variation than dichlorodimethylhydantoin or chloramine-T.

Chlorine dioxide does not ionize in water, and therefore its bactericidal efficiency remains essentially constant over the normal range of pH value in natural water (Ridenour and Ingols, 1947; Bernarde et al., 1965). At pH 8.5, equivalent concentrations of chlorine dioxide and hypochlorite produce an equivalent amount of *E. coli* kill in 15 and 300 seconds, respectively. At pH 6.5, the two compounds were almost equally effective at equivalent concentrations. Maintaining solutions of acidified sodium chlorite in a pH range of 2.3 to 3.2 is critical for maximum effectiveness.

Berman and Hoff (1984) studied the effect of chlorine, chlorine dioxide, and monochloramine on viral preparation at 5°C and pH 6 and 10. The results indicated that 0.5 ppm available chlorine as sodium hypochlorite inactivated virions in less than 15 seconds at pH 6, resulting in 4 log₁₀ reduction but not at pH 10. With chlorine dioxide, however, 0.5 ppm was more efficient at pH 10 than at pH 6, again exhibiting a 15-second inactivation. Monochloramine at 10 ppm concentration and pH 8 required more than 6 hours to produce an equivalent inactivation of virions. Harakeh et al. (1985) demonstrated that *Y. enterocolitica* and *Klebsiella pneumoniae* can be more easily controlled by chlorine dioxide when the organisms are prepared under optimum laboratory conditions than those grown in natural aquatic environments. The authors recommend the use of the naturally resistant organisms for determining the efficacy of disinfectants.

It is interesting that exposure and subsequent adaptation of *Salmonella* to an acidic environment has been found to sensitize the organism to treatment with hypochlorous acid (Leyer and Johnson, 1997). Similar results were demonstrated in the case of *L. monocytogenes*, in which alkali stress resulted in increased susceptibility to chlorine inactivation (Taormina and Beuchat, 2001).

TABLE 16.7
Effect of pH on Destruction of Various Microorganisms by Hypochlorite

Organism	Hypochlorite Concentration (ppm)	pH of Solution	Average Number of Surviving Microorganisms ^a				
			15 s	30 s	60 s	120 s	300 s
<i>Escherichia coli</i> (initial count = 3.7×10^6 ml ⁻¹)	3	8.5	11	0	0	0	0
	3	5.1	0	0	0	0	0
	6	8.6	2	0	0	0	0
	6	5.1	0	0	0	0	0
<i>Streptococcus lactis</i> (initial count = 6.8×10^5 ml ⁻¹)	3	8.4	TNTC ^b	TNTC	TNTC	TNTC	TNTC
	3	5.0	0	0	0	0	0
	6	8.4	38	5	0	0	0
	6	5.0	0	0	0	0	0
<i>Pediococcus cerevisiae</i> (initial count = 8.5×10^6 ml ⁻¹)	3	8.4	TNTC	TNTC	TNTC	TNTC	TNTC
	3	5.1	0	0	0	0	0
	6	8.5	TNTC	496	19	127	20
	6	5.1	0	0	0	0	0
<i>Saccharomyces cerevisiae</i> (initial count = 4.7×10^5 ml ⁻¹)	3	8.6	TNTC	TNTC	TNTC	61	0
	3	5.2	TNTC	66	0	0	0
	6	8.5	TNTC	TNTC	14	0	0
	6	5.3	81	2	0	0	0

^a Survivors after 15 to 300 seconds of exposure.

^b TNTC, too numerous to count.

Source: Adapted from Hays et al. (1967).

TABLE 16.8
Effect of pH and Temperature on Inactivation of
***Bacillus metiens* Spores by Hypochlorite (25 ppm**
Available Chlorine from Ca(OCl)₂)

Temperature (°C)	pH	Time (min) for 99% Destruction
20	12.9	465
20	10.0	121
20	9.0	20
20	8.0	5
20	7.0	3.6
20	6.0	2.5
20	10.0	121
30	10.0	65
35	10.0	39
50	10.0	9

Source: Adapted from Rudolph and Levine (1941).

Effect of Temperature

A significant amount of work has been published relating to the effect of temperature on the antimicrobial activity of chlorine (Trueman, 1971; Dychdala, 2001). At high concentrations of chlorine, the temperature effect of chlorine is not readily apparent; at low concentrations the effect is magnified. This is particularly true in vegetative cells. Johns (1954) found essentially no difference in the rate of kill for hypochlorite against *Micrococcus pyogenes* var. *aureus*, *E. coli*, or *Pseudomonas aeruginosa* at approximately 10 ppm and temperatures of 5°C, 20°C, and 45°C. Likewise, Tuncan (1993) demonstrated no influence on the efficacy of chlorine (25 ppm) on *Listeria* species at a temperature range of 2°C to 25°C. These results also indicated that although hypochlorite is sensitive to temperature effects, it is significantly less sensitive than the other commonly used sanitizers. Rudolf and Levine (1941) studied the effect of temperature on the sporicidal activity of hypochlorite. The results, presented in Table 16.8, show a pronounced temperature effect on the spores of *B. metiens*.

Temperature sensitivity data for the organic chlorine compounds are not readily available. Johns (1954) found that a dichlorodimethylhydantoin product was more temperature sensitive than hypochlorites, especially against *P. aeruginosa*. A decrease in temperature from 45°C to 5°C necessitated a fourfold increase in organic chlorine content to achieve the same rate of kill. Bernarde et al. (1967a) evaluated the effect of temperature on chlorine dioxide disinfection. These authors observed a substantial temperature effect over the range of 5°C to 32°C; however, no direct comparison to temperature effects on hypochlorite was established. Driedger et al. (2001) showed that the rate of *C. parvum* inactivation decreased with decreasing temperature following treatment with monochloramine.

Effect of Organic Material

The effect of organic matter on the bactericidal efficacy of chlorine compounds is well documented (Kotula et al., 1997; Dychdala, 2001). The type of organic material present and the amount of this material of course influence the extent to which efficacy is depressed (Hekmati and Brakely, 1979). The data presented in Table 16.9 illustrate the effect of bovine lean or porcine fat meat tissue on residual chlorine concentrations following treatment up to 96 minutes. Differences between tissue

TABLE 16.9
Influence of Meat Tissue Type and Time of Exposure on Free Available Chlorine Levels as Hypochlorite

Organic Load	FAC ^a (ppm)	Number of Cubes ^b	Percent Residual Chlorine Concentration at Exposure Time (min) of:								
			0.5	4	8	16	32	48	64	80	96
Bovine lean	50	1	100	100	100	80	80	60	60	60	60
		8	100	80	80	60	60	40	34	28	24
	100	1	100	90	90	90	80	70	70	60	50
		8	100	70	60	60	50	50	40	40	40
Porcine fat	50	1	100	100	100	100	100	100	100	100	100
		8	100	100	100	100	100	80	80	80	60
	100	1	100	100	100	100	100	100	100	100	90
		8	100	90	90	90	90	90	90	80	80

^a FAC, free available chlorine.

^b Meat tissue cubes (1.5 cm³).

Source: Adapted from Kotula et al. (1997).

types may be attributed to the binding of free chlorine by amino groups in the proteinaceous lean tissue. Johns (1954) compared the activity of various sanitizing solutions in the presence of 0.5% skim milk at different temperatures. The organic chlorine compound dichlorodimethylhydantoin appeared to be less sensitive to the addition of organic material than the hypochlorite solution. Bloomfield and Miles (1979b) recently compared the bactericidal effect of sodium hypochlorite and sodium dichloroisocyanurate in the presence of organic material and observed the organic chlorine compound to be less affected by organic material. The authors found equivalent activity of the two chlorine sources in the absence of organic material, with a fairly abrupt change in efficacy as the milk concentration was increased from 0.5% to 2.0%. The sporicidal activity of sodium dichloroisocyanurate, however, has been demonstrated to be significantly decreased by the addition of 2% blood in treatment solutions (Coates, 1996). ClO_2 is a strong oxidizing agent and, in contrast to hypochlorite, does not react with ammonia and other nitrogenous compounds and is in general not as susceptible to the effect of organic matter (White, 1972). Reactions between ClO_2 and other biomolecules are reviewed by Knapp and Battisti (2001). Lillard (1979) found that chlorine dioxide was significantly more effective than chlorine in reducing the aerobic and fecal coliform count in poultry processing water.

Effect of Water Hardness

Water hardness as such does not exert a significant effect on the antimicrobial activity of chlorine. In many cases, however, hard water causes an upward drift in pH, thereby reducing chlorine effectiveness by a pH effect (Hays et al., 1967; Mosley et al., 1976).

TOXICITY

Since 1976 when chloroform, a trihalomethane (THM) and a major disinfection by-product, was shown to be carcinogenic in rodents, concerns regarding the use of chlorine as a drinking water disinfectant have received much attention (Boorman et al., 1999). THMs are formed on the reaction of chlorine with organic material (principally humic acids) present in water. In 1979, the EPA reacted to the safety concerns of disinfection by-products by promulgating regulations limiting the permissible levels of THMs in drinking water. Following an extensive study of public water supplies throughout the United States and the nature, source, and control of suspected carcinogenic substances, a new maximum level of THMs in drinking water of 80 ppb has been established (Code of Federal Regulations, 1998). Water companies are required to monitor for THM levels in drinking water, and if present, these chemicals must be removed by a specific treatment that is technically and economically feasible because exposure to THM in drinking water poses a risk to human health.

A procedure was developed to remove organic contaminants from drinking water by granular activated carbon columns. The best approach would be to remove THM precursors from the water, which would preclude the formation of THM during the chlorination of drinking water. This was a dilemma for the regulatory and technical community, which realized that there are not many substitutes available for chlorination. Without chlorination, the consequences of microbial contamination and health hazards would be enormous, and with chlorination, the concern shifts to the formation of THM, which is unacceptable because its toxicity poses a human health risk.

Pieterse (1988), in discussing trihalomethane formation in drinking water as a result of chlorination, conceded that THMs are toxic and carcinogenic to animals at high concentrations and can also be mutagenic. The most important priority stated was the removal of microbiological contamination from drinking water to ensure public health safety. However, he cautioned the regulatory agencies to be responsible and realistic in their approach to establishing regulations for this purpose.

Fukayama et al. (1986) reviewed the potential health risks associated with the use of chlorine with some food products. The authors showed several of the chemical reactions of chlorine and chlorine dioxide with basic food components and explained the residue levels found on some of

these foods. Solutions containing chlorine should not be combined with acid solutions or adjusted to low pH because of the formation of highly toxic chlorine gas.

Chlorine dioxide has been suggested as an alternative to chlorine for water disinfection; however, health risks are involved with its use also. Bull (1980) recently reviewed this subject and concluded that chlorine dioxide and its inorganic reaction products (specifically chlorite) may present a higher risk from an acute toxicity standpoint than chlorine. Also, because it is a very reactive compound, chlorine dioxide cannot be shipped and transported safely, so it is mostly prepared at the place of application. Some methods of preparation include chlorination of sodium chlorite, acidification of sodium chlorate or sodium chlorite, and electrolytic generation. The procedure of preparing chlorine dioxide at the plant site is the most practical and safe way of generating this compound for immediate consumption purposes, including the disinfection of water or wastewater.

IODOPHORS

Iodophors are mixtures of iodine and surface-active agents that act as carriers and solubilizers for the iodine. In 1949, Shelanski discovered that polyvinylpyrrolidone (PVP) and surface-active agents could be used to solubilize iodine and form complexes that retained germicidal activity and at the same time reduced the undesirable properties of the iodine (Davis, 1962). These iodophors are able to solubilize nearly 30% of their weight of iodine and, subsequently, release available iodine when a concentrated solution is diluted.

Today the term “iodophor” refers to two basic types of aqueous iodine preparations: (1) reaction of iodine with PVP and (2) reaction of iodine with surfactant molecules. The latter type, iodine combined with surfactant, is the most important type of compound with respect to food industry use. The iodophors are primarily produced from polyethoxylated nonylphenol or polyol, which is a block copolymer of propylene and ethylene oxide. Various other surfactants, including anionics, cationics, amphoteric, and other nonionics, have also been used (Batey, 1976). In general, the alkylphenoxy polyglycol ethers have proved to be the most suitable of the nonionic surfactants, with some complexing up to 28% iodine (Bartlett and Schmidt, 1957).

The actual structure or nature of the interaction between the iodine and the surfactant has not been clearly defined. It is known, however, that the iodine is bound in micellar aggregates in the carrier and that on dilution the micelles are dispersed and the linkage of the iodine is progressively reduced (Twomey, 1968, 1969). In some cases, binding of the iodine to the carrier is so tenacious that dilution of the concentrate actually produces a more cidal preparation than the concentrate itself (Favero, 1982; Jerusik, 1981). The function of the iodine–surfactant complex is (1) to increase solubility of iodine, (2) to provide a sustained-release reservoir of the halogen, and (3) to reduce the equilibrium concentration of free molecular iodine (Gottardi, 2001).

The forms of iodine present in aqueous solution as a function of pH and the relative bactericidal activity of the various chemical species of iodine are illustrated in Table 16.10.

MECHANISM OF ACTION

The exact mechanism for the antimicrobial activity of iodophors has not been as widely studied and is not as clearly defined as that of the chlorine compounds (Gottardi, 2001). McCulloch (1932) believed that iodine destroyed microorganisms by direct halogenation of proteins to form salts. Shakashio (1969) and colleagues (1968) reviewed the literature with respect to the mechanism of action and reported that oxidation of sulfhydryl groups and substitution of tyrosyl and histidyl groups on proteins were primary effects. In their work, these authors found that iodine inactivated certain sulfhydryl enzymes and that this inactivation was the result, at least in part, of oxidation of essential sulfhydryl groups. This appeared to be true for alcohol dehydrogenase. Addition of iodine

TABLE 16.10
Relation between pH and Bactericidal Efficacy of Iodine

pH	Major Ionic Species Present	Relative Bactericidal Activity ^a
Acid	I ₂	+++
Intermediate	I ₂	+++
	HIO	++
Alkaline	IO ⁻	+
	IO ⁻	+
	IO ₃ ⁻	-
	I ⁻	-
	I ₃ ⁻	-

Note: A + + +, most active; + +, moderately active; +, slightly active; -, inactive.

Source: Adapted from Wilson et al. (1960).

to unsaturated fatty acids is thought to lead to a change in the physical properties of lipids causing membrane immobilization, which results in the loss of intracellular materials. Less is known regarding the virucidal action of iodophors. Inactivation of enveloped viruses has been attributed to conformational changes to the protein coat (Brion and Silvestein, 1999).

ANTIMICROBIAL ACTIVITY

Iodine, like chlorine, is bactericidal over a fairly broad pH range against a wide spectrum of bacteria, fungi, viruses, and protozoa, including bacterial and fungal spores (Gottardi, 2001). In many cases, the iodophors are effective at much lower concentrations than chlorine (Gershenfeld and Witlin, 1955; Trueman, 1971). In an extensive study by Gray and Hsu (1979), iodophors were shown to be equal and, under some conditions, superior to chlorine for the inactivation of *Vibrio parahaemolyticus*. Lindsey and von Holy (1999) investigated the effectiveness of an iodophoric preparation at 35 ppm iodine to reduce populations of planktonic and sessile *Bacillus subtilis* and *Pseudomonas fluorescens*. The iodophor performed as well as the peracetic acid- and chlorohexidine-based sanitizers also analyzed in inactivating planktonic *P. fluorescens*. Its efficacy in killing *P. fluorescens* attached to stainless steel as well as both planktonic and sessile *B. subtilis* was limited.

According to Russell (1990), the sporicidal activity of iodine (iodophor) is dependent on the pH and the concentration and the best activity was achieved in acid pH and at a higher concentration than normally used for general sanitation.

Iodophors appear to be slightly more effective against yeasts than chlorine (Hays et al., 1967; Mosley et al., 1976), although this observation varies with the genus of yeast evaluated. Mosley et al. (1976) found the genus *Candida* to be significantly more susceptible to iodophor than hypochlorite when compared to an equivalent concentration of active ingredients (Table 16.12).

The inactivation of bacteriophage by iodophors has been documented, although they are generally slower and require higher concentrations to produce the same effect as hypochlorites (Watkins et al., 1957; Sing et al., 1964a, b). McCoy and Irwin (1974) compared the activity of various commercial disinfectant preparations against *E. coli* bacteriophage ϕ X174 and found only chlorine and iodine to be effective. The activity of iodophors against mammalian viruses has also been demonstrated (Bartlett and Schmidt, 1957; Gottardi, 2001).

Very little information is available regarding the efficacy of iodophors in inactivation coccidian protozoa. Wilson and Margolin (1999) treated oocysts of *Cryptosporidium parvum* with 10% povidone-iodine. Percent reductions in excystation were 47%, 60%, and 70% after 10 minutes,

TABLE 16.11
Inactivation of Various Microorganisms by Iodophors

Organism	Concentration ^a (ppm)	pH	Temperature (°C)	Exposure Time	Inactivation (%)	Reference
<i>Escherichia coli</i>	6	6.9	25	30 s	>99.999	Hays et al. (1967)
<i>Streptococcus lactis</i>	6	5.0	25	60 s	>99.999	Hays et al. (1967)
<i>Lactobacillus plantarum</i>	6	5.0	25	120 s	>99.999	Hays et al. (1967)
<i>Pediococcus cerevisiae</i>	6	5.0	25	100 s	>99.999	Hays et al. (1967)
<i>Saccharomyces cerevisiae</i>	6	6.8	25	15 s	>99.999	Hays et al. (1967)
<i>Candida</i> sp.	25	3.1	25	15 s	>99.999	Mosley et al. (1976)
Yeasts	25	4.4	15	8 s	90.000	Braithwaite (1973)
<i>Bacillus subtilis</i>	100	2.3	21	240 min.	99.000	Cousins and Allan (1967)
<i>Bacillus cereus</i>	100	2.3	21	12 min.	99.000	Cousins and Allan (1967)
<i>Bacillus macerans</i>	500	6.5	25	>60 min.	99.990	Seeger (1978)
<i>Bacillus stearothermophilus</i>	500	6.5	25	>60 min.	99.990	Seeger (1978)
PA 3679	500	6.5	25	60 min.	99.990	Seeger (1978)

^a Titratable iodine.

90 minutes, and 10 hours of exposure to the iodophor, respectively. Infectivity, however, was not influenced by treatment.

FACTORS AFFECTING ACTIVITY

The degree to which environmental factors, such as pH, temperature, organic material, and water hardness, influence the antimicrobial activity of iodophors varies considerably with the specific iodophor. It is therefore imperative that the actual iodophor composition be considered and that statements addressing iodophors in general terms be avoided.

Effect of pH

The efficiency of the iodophors as germicides is directly influenced by pH. Commercial iodophor compositions are generally formulated to deliver an acid pH at use dilution. An abundance of information in the scientific and trade literature implies that iodophors are only germicidal in the acid pH range (pH 2.0 to 5.0). A survey of the literature and actual testing in our laboratory has shown that iodophors are also effective in the pH range 5.0 to 8.0. The data presented in Table 16.13 indicate that an increase of almost two pH units (pH 5.0 to 7.0) alters the effectiveness of the iodophor, especially at low concentrations. However, this effect compared to pH alteration effects of hypochlorite (Table 16.7) is relatively minimal. An iodophor composition containing butoxy-polypropoxypolyethoxyethanol iodine as the active ingredient was evaluated in our laboratory using

TABLE 16.12
Effect of Water Hardness on Germicidal Activity of Various Sanitizers^a

Germicide	Concentration (ppm)	Test Water	pH Germicide Solution	15 s	30 s	60 s
<i>Salmonella Derby</i> ^b						
Iodophor B	25	Distilled	2.50	TNTC ^c	11	0
	25	USDA ^d	2.70	TNTC	TNTC	TNTC
Iodophor C	25	Distilled	2.90	0	0	0
	25	USDA	6.25	TNTC	TNTC	TNTC
Iodophor E	25	Distilled	3.25	0	0	0
	25	USDA	6.70	2	0	0
Hypochlorite	25	Distilled	6.25	0	0	0
	25	USDA	7.20	0	0	0
QAC A	100	Distilled	2.80	TNTC	TNTC	TNTC
	100	USDA	3.65	TNTC	TNTC	TNTC
QAC B	100	Distilled	5.20	0	0	0
	100	USDA	7.70	TNTC	TNTC	TNTC
<i>Escherichia coli</i> ^e						
Iodophor B	25	Distilled	2.39	TNTC	TNTC	TNTC
	25	USDA	2.50	TNTC	TNTC	TNTC
Iodophor C	25	Distilled	2.89	TNTC	3	0
	25	USDA	6.25	TNTC	TNTC	TNTC
Iodophor E	25	Distilled	3.20	3	0	0
	25	USDA	6.90	TNTC	1	0
Hypochlorite	25	Distilled	7.75	0	0	0
	25	USDA	8.60	0	0	0
QAC A	200	Distilled	2.60	TNTC	TNTC	0
	200	USDA	2.65	TNTC	0	0
QAC B	200	Distilled	5.22	0	0	0
	200	USDA	7.45	TNTC	17	3
<i>Candida</i> ^f sp.						
Iodophor B	25	Distilled	2.71	1	0	0
	25	USDA	2.60	TNTC	0	0
Iodophor C	25	Distilled	2.80	0	0	0
	25	USDA	6.00	TNTC	TNTC	TNTC
Iodophor E	25	Distilled	3.10	0	0	0
	25	USDA	6.80	0	0	0
Hypochlorite	25	Distilled	6.50	TNTC	TNTC	56
	25	USDA	8.10	TNTC	TNTC	TNTC
QAC A	200	Distilled	2.65	TNTC	TNTC	TNTC
	200	USDA	3.50	TNTC	TNTC	4
QAC B	200	Distilled	5.90	20	0	0
	200	USDA	8.81	37	0	0

^a Iodophor B and C, nonionic wetting agent combined with iodine; iodophor E, butoxypolypropoxypolyethoxy-ethanoliodine complex with 6.5% phosphoric acid; QAC A, n-alkyldimethylethylbenzylammonium chloride with 30% phosphoric acid; QAC B, n-alkyldimethyldichlorobenzylammonium chloride.

^b Initial number 29×10^9 ml⁻¹.

^c TNTC, too numerous to count.

^d U.S. Department of Agriculture, 500 ppm (CaCO₃).

^e Initial number 8×10^9 ml⁻¹.

^f Initial number 3×10^8 ml⁻¹.

Source: Adapted from Mosley et al. (1976).

TABLE 16.13
Effect of pH on Destruction of Various Microorganisms by an Iodophor^a

Organism	Iodine Concentration (ppm)	pH of Solution	Average Number of Surviving Organisms ^b				
			15 s	30 s	60 s	120 s	300 s
<i>Streptococcus lactis</i> (initial count = 6.8×10^5 ml ⁻¹)	3	7.1	TNTC ^c	TNTC	2200	287	0
	3	5.0	TNTC	TNTC	520	2	0
	6	6.8	TNTC	720	14	0	0
	6	5.0	TNTC	412	0	0	0
<i>Pediococcus cerevisiae</i> (initial count = 8.5×10^6 ml ⁻¹)	3	7.2	TNTC	TNTC	TNTC	TNTC	TNTC
	3	5.1	TNTC	TNTC	TNTC	315	0
	6	6.9	TNTC	980	4	0	0
<i>Lactobacillus plantarum</i> (initial count = 2.8×10^5 ml ⁻¹)	6	5.0	TNTC	TNTC	700	0	0
	3	7.0	TNTC	TNTC	TNTC	TNTC	TNTC
	3	5.1	TNTC	TNTC	TNTC	143	0
	6	6.7	TNTC	TNTC	0	0	0
	6	5.0	TNTC	TNTC	328	0	0

^a Nonylphenoxy poly(ethyleneoxy)ethyl alcohol-iodine complex with 25% phosphoric acid.

^b Survivors after 15 to 300 seconds of exposure.

^c TNTC, too numerous to count.

Source: Adapted from Hays et al. (1967).

TABLE 16.14
Inactivation of *Staphylococcus aureus* and *Escherichia coli* by an Iodophor^a under Various Conditions of pH and Organic Load

pH	Presence of 0.5% Skim Milk	Inactivation Results (%) ^b	
		<i>S. aureus</i> ^c	<i>E. coli</i> ^d
3.3	–	>99.999	>99.999
3.3	+	>99.999	>99.999
6.0	–	>99.999	>99.999
6.0	+	99.99	99.998
9.0	–	>99.999	>99.999
9.0	+	30.233	42.708

^a Mikrokylene, butoxypropoxypolyethoxyethanol–iodine, at 12.5 ppm titratable iodine.

^b AOAC germicidal and detergent sanitizer test, 30 s exposure (1980).

^c Initial count = 7.4×10^7 .

^d Initial count = 9.7×10^7 .

Source: Fredell and Givins (1981).

the Association of Official Analytical Chemists (AOAC, 1984) germicidal and detergent sanitizer test. In this instance, a 5- \log_{10} reduction was obtained using 12.5 ppm available iodine over a pH range of 3.3 to 9.0 (Table 16.14). Lopes (1986) evaluated iodophor at 12.5 and 25 ppm titratable iodine and showed a 5- \log_{10} reduction of *L. monocytogenes* at both concentrations. In the same experiment he found that *S. Typhimurium* was slightly more resistant to iodine and produced the required 99.999% kill in 30 seconds only with 25 ppm titratable iodine concentration. Although many iodophor compositions are highly germicidal over a broad pH range, certain compositions, either by virtue of the type of surfactant used in the composition or the method of manufacture, are quite sensitive to pH. Neutral and alkaline pH definitely affect the stability of iodine in solution and thus the germicidal activity. The data of Mosley et al. (1976) are reproduced in Table 16.12 and show a wide variation in the effect of pH on different iodophors.

Chambers et al. (1952) studied the effect of pH and temperature on the antimicrobial efficacy of iodine (not an iodophor) and found that, at a temperature of 20°C to 26°C, the effect of temperature was minimal; at 2°C to 5°C, slightly lower concentrations were required for kill at pH 6.5 compared to pH 9.15.

The presence of organic matter also influences the sensitivity of iodophor to pH effects. This is well illustrated in Table 16.14. At pH 9.0 in distilled water, this product passed the AOAC germicidal and detergent sanitizing test, but at pH 9.0 in the presence of 0.5% skim milk, the total amount of kill in 30 seconds was less than 50%.

Effect of Temperature

Johns (1954) studied the effect of temperature on the antibacterial activity of iodophors. In this study, the efficacy of the iodophors against *E. coli* at 5°C was significantly lower than at 20°C or 45°C. Data presented for *P. aeruginosa* indicated that this dramatic temperature effect may not be observed in all cases. Iodophors indeed appear to be more temperature sensitive than hypochlorites. A temperature of 120°F or higher affects the stability of iodine in solution because iodine has a high vapor pressure. In the range of general use temperatures in the food industry, however, this effect is minimal.

Effect of Organic Matter

Iodophor sanitizing solutions do not lose antimicrobial efficacy as rapidly as chlorine in the presence of organic matter. This is especially true at low pH (Davis, 1962). At higher pH an organic matter effect becomes readily apparent, as shown in Table 16.14. At low temperature, the effect of organic matter on activity may also be magnified (Johns, 1954). Iodophors are generally considered less susceptible to organic matter than chlorine and more susceptible to organic matter than quaternary ammonium compounds (Harper, 1980). However, in a study by Miner et al. (1975), the iodophors were shown to be slightly more resistant to organic neutralization than quaternary ammonium compounds at manufacturers' recommended use concentrations. These authors also compared various disinfectants based on an equal level of active ingredients. The iodophors exhibited a markedly higher resistance to organic material. The work of Johns (1954) tends to support the conclusions of Miner et al. (1975), especially at normal use temperatures.

Effect of Water Hardness

Iodophors are generally considered more adversely affected by water hardness than hypochlorites. The degree to which hard water depresses the activity of iodophors is highly dependent on the specific type of iodophor being evaluated. Mosley et al. (1976) studied the effect of hard water on the antimicrobial activity of commercial iodophor preparations. The data presented in Table 16.12 indicate that a hard water effect independent of pH effect can be demonstrated for iodophor B. In this study, iodophor E was only minimally affected by 500 ppm hard water as CaCO_3 . Based on previous discussion in this chapter, one can speculate that the hard water effects would be magnified by the presence of organic material.

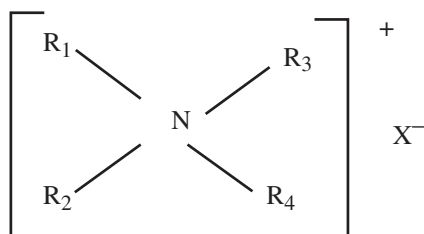
TOXICITY

There is an abundance of information in the early literature (Gershenfeld, 1957) with regard to iodine toxicity. Iodine solutions have a long history of direct use on skin and open wounds. Acute poisoning resulting from iodine ingestion has been reported; however, these cases involved ingestion of gram levels of iodine (Gosselin et al., 1976). Lazarus (1954) reported a median lethal dose (LD_{50}) of 4.5 ml/kg in white rats for an iodophor solution containing 10% iodine and 90% nonionic surfactant of the alkylphenoxy polyglycol ether type. The use of iodophor sanitizers, according to the manufacturers' recommendations, presents no readily apparent hazard to human health.

SURFACE-ACTIVE AGENTS

QUATERNARY AMMONIUM COMPOUNDS

The term "quaternary ammonium compound" (QAC) in essence defines a group of chemical substances that are synthesized by a nucleophilic substitution reaction of tertiary amines with a suitable quaternizing agent, such as an alkyl halide or benzyl chloride. The basic chemical structure can be depicted as follows:



where R_1 , R_2 , R_3 , and R_4 represent covalently bound alkyl groups, which may be alike or different, substituted or unsubstituted, saturated or unsaturated, branched or unbranched, cyclic or acyclic, or aromatic or substituted aromatic groups. In addition, the alkyl groups may contain ester, ether, or amide linkages. The nitrogen atom plus the attached R groups form the cation. The anion, most often chloride, is bound to the nitrogen by an ionic bond. The QAC family is diverse and generalizations regarding antimicrobial efficacy and environmental influence are extremely difficult.

The QACs were originally developed as aqueous solutions to be used as disinfectants. Today, many formulations are classified as detergent sanitizers in which quaternary compounds are combined with nonionic surfactants or other detergent builders. These formulations are desirable for many applications because they are germicidal, have good physical and chemical stability and detergent action, and are effective over a fairly broad pH range. They are also colorless, relatively odorless, noncorrosive, and of relatively low toxicity to most common materials of construction. Quaternary ammonium compounds approved as no-rinse sanitizers for food contact surfaces include n-alkyldimethylbenzylammonium chloride and di-n-alkyldimethylammonium chlorides and n-alkyldimethylethylbenzylammonium chlorides. Mixtures of one or more of these occur in many products approved by EPA as no-rinse sanitizers.

MECHANISM OF ACTION

The mode of action of QACs and the chemical properties of the cationic surfactants are directly related. These properties include the following: (1) reduction in surface tension; (2) attraction to negatively charged entities, such as proteins and bacteria; (3) formation of ionic aggregates, with resultant changes in conductivity, surface tension, and solubility; and (4) a denaturing effect on proteins (Petrocci, 1983). Cell membrane disorganization and denaturation of essential proteins (Baker et al., 1941) as well as release of nitrogenous and phosphorus-containing cell constituents (Hotchkiss, 1946) are mechanistic effects of quaternary compounds reported by some of the early investigators. Hugo (1965) summarizes several possible modes of action for these compounds:

1. Direct influence on proteins
2. Effects on metabolic reactions
3. Effects on cell permeability
4. Impact on glycolysis reactions
5. Effect on enzymes that maintain a dynamic cytoplasmic membrane

Hugo concluded that cytoplasmic membrane damage was the mechanism that best fit the majority of research on the mechanism of inactivation by these compounds.

The importance of chain length of the various R groups on antimicrobial efficacy cannot be overemphasized. Cutler et al. (1966) studied the antimicrobial activity of homologous series of alkyldimethylbenzylammonium chlorides and found the highest activity for those containing alkyl groups in the range of C_{12} to C_{16} , C_{14} being maximum. Other QACs may display a slightly different spectrum of activity for homologous series. However, most commercial n-alkyldimethylbenzylammonium chloride compounds contain a high percentage of C_{12} to C_{16} alkyl groups. The dialkyl quaternary compounds typically contain actyl or decyl groups.

ANTIMICROBIAL ACTIVITY

Quaternary ammonium compounds have antimicrobial activity against bacteria yeasts, molds, protozoa, and viruses. Generalizations on their antimicrobial activity are difficult because of the heterogeneity of available compounds. The antimicrobial efficacy of various QACs against Gram-negative bacteria, Gram-positive bacteria, and fungi is compared in Table 16.15. The variation in efficacy among the different compounds is evident, as well as significantly greater activity against Gram-positive organisms compared to the Gram-negative organisms such as *E. coli* and *Pseudomonas*

TABLE 16.15
Minimum Inhibitory Concentration (ppm) of Quaternary Ammonium Compounds for Several Bacteria and Fungi^a

Compound ^b	Organism					
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Aspergillus niger</i>	<i>Trichoderma viride</i>
A. Benzalkonium chloride	200	300	3	4	60	80
Monoquaternalaries R ₄ N ⁺ N ⁻ (one R group is fatty acid)						
B. Dodecyltrimethylammonium chloride	500	500	5	5	500	500
C. Tetradecyltrimethyl-ammonium chloride	150	100	1.5	5	50	150
D. Hexadecyltrimethyl-ammonium chloride	5000	5000	5	5	500	150
E. Dodecylbenzyl-dimethyl-ammonium chloride	750	750	2	2	75	75
Monoquaternalaries R ₄ N ⁺ X ⁻ (two R groups are fatty acids)						
F. Dioctyldimethylammonium chloride	40	75	20	20	225	75
G. Didecyldimethylammonium chloride	225	750	<0.7	7	75	20
H. Ditetradecyldimethyl-ammonium chloride	2250	>2250	225	750	2250	2250
Monoquaternalaries R ₄ N ⁺ X ⁻ (three R groups are fatty acids)						
I. Tri(octyldecyl)methyl-ammonium chloride	500	500	3	5	150	100
J. Tridodecylmethyl-ammonium chloride	>5000	>5000	500	1500	1500	1500

^a Values in ppm are for bacteriostatic or fungistatic activities, not cidal.

^b Compounds studied were highly purified. The name of the major alkyl group(s) present is listed.

Source: Adapted from Hueck et al. (1966).

fluorescens. There are reports in literature of Gram-negative bacteria with uncommonly high tolerance to QACs (Adair et al., 1969; Washam et al., 1976; Ehrenschrantz et al., 1980; Stickler and Thomas, 1980). Certain Gram-negative bacteria, however, can be inactivated using quaternary concentration equivalent to those effective against Gram-positive organisms. Lopes (1986), in his comparative tests, showed that QACs were effective against *L. monocytogenes* at 100 and 200 ppm, and a minimum of 200 ppm was required to control *S. Typhimurium*.

The fungicidal activity of QACs is well documented (Hueck et al., 1966; Petrocci, 1983). Greene and Petrocci (1980) indicated fungicidal activity of a quaternary ammonium formulation containing a 50:50 mixture of n-alkyl- (60% C₁₄, 30% C₁₆, 5% C₁₂, 5% C₈) dimethylbenzylammonium chloride and n-alkyl- (68% C₁₂, 32% C₁₄) dimethylethylethylbenzylammonium chloride against *Trichophyton mentagrophytes* at 488 ppm. In general, the fungicidal activity of QACs is quite variable and depends on the specific quaternary compound used, the use conditions, and the genus and species of the target organism. Earlier researchers observed that QACs possess sporicidal activity (Russell, 1982). These studies have since been refuted, and although the QACs may be sporostatic by virtue of inhibiting germination or outgrowth of spores, they are not considered sporicidal (Hays, 1956; Russell, 1971; Mosley et al., 1976; Cook and Pierson, 1983; Russell et al., 1985).

The virucidal activity of QACs has been investigated by several authors (Klein and Deforest, 1963; Armstrong and Froelich, 1964; Stols and Veldstra, 1965). They appear to be fairly effective at medium concentrations against enveloped viruses but are relatively ineffective even at high concentrations against nonenveloped viruses, which have no affinity for lipids. Watkins et al. (1957) found that QACs exhibit virucidal effects on bacteriophages of lactic acid bacteria. Quaternary compounds are, however, inhibitory to lactic acid bacteria at fairly low levels and their use as sanitizers in fermented food plants should be carefully controlled to avoid residuals that could affect acid production. According to Lembke and Teuber (1981), although 0.5% alkylbenzyl dimethylammonium chloride and 0.5% N-cetylpyridinium chloride inactivated several strains of bacteriophages, this inactivation required an extended time of 60 minutes. Incorporation of ethylenediaminetetraacetic acid (EDTA) into the quaternary formulation enhanced the virucidal capacity. Sing et al. (1964a, b) compared the virucidal activities of several sanitizing compounds when applied as aerosols. Their work indicated that QACs were not effective, even on incorporation of 200 ppm EDTA into 2000 ppm QACs.

FACTORS AFFECTING ACTIVITY

The antimicrobial activity of QACs is directly affected by the environmental conditions present at the time of contact with the target microorganisms. The pH, temperature, organic matter, and water hardness may all combine to affect ultimate activity. Because of the diversity of QACs available, general statements regarding QACs are even more difficult than for chlorine compounds or iodophors.

Effect of pH

Much of the early research that dealt with the effect of hydrogen ion concentration on the antimicrobial activity of QACs suggests that maximum efficacy is exhibited in the alkaline pH range (Soike et al., 1952). In most cases this is true; however, the results obtained by Soike et al. (1952) indicate that bacterial species vary considerably with respect to effective pH for maximum bactericidal activity. In their study, *P. aeruginosa* possessed increased resistance to four different QACs at pH 7 and above and was most susceptible in the acid pH range. For *Micrococcus caseolyticus*, the antimicrobial activity was highest at pH 7 and above, although resistance was observed at pH 3 to 4. This pattern of altered resistance based on the pH of the test solution was further complicated by the results obtained with *E. coli*, for which peaks of resistance were observed below pH 6 and

at approximately pH 8.0. *E. coli* appears to be most susceptible to inactivation at pH 7.0. Salton (1950) observed similar alkaline conditions. Soike et al. (1952) suggested that the marked variation in the rate of destruction with alternation of pH is the result of factors associated with the cell rather than the specific effects of pH on the activity of the QAC molecule itself. The results obtained by Mosley et al. (1976), which are presented in Table 16.12, generally are indicative of lower activity at high pH; however, these results are complicated by the water hardness effect.

Effect of Temperature

There is general agreement with regard to the effect of temperature on the biocidal activity of QACs: the activity of QACs is enhanced with increased temperature (Johns, 1954; Petrocci, 1983). This temperature effect is more pronounced for quaternary compounds than for traditional hypochlorite or iodophor sanitizers. Tuncan (1993) showed that several *Listeria* species are significantly more tolerant to a QACs at temperatures of 2°C and 7°C compared to room temperature.

Effect of Organic Matter

There are conflicting reports in the literature regarding the effect of organic material on the antimicrobial efficacy of QACs. This is in large part a result of the variability of QACs themselves and also depends on the type of organic material used in the study. Hornung (1935) and Schneider (1935) found that the presence of 10% to 16% serum did not depress the antimicrobial activity to any appreciable degree. Curran and Evans (1950) reported a significant reduction in sporicidal activity by traces of skim milk. Quisno et al. (1946) found that the presence of 0.2% agar depressed the lethal activity of four different QACs formulations to such an extent that a three to six times greater concentration of QACs was required to achieve destruction equivalent to the control. Miner et al. (1975) studied the effect of organic soil neutralization on the activity of several disinfectant compositions. When they adjusted the percentage of active ingredient of each disinfectant to an equal concentration of 0.2%, iodophors were more resistant to neutralization by yeast than QACs. At recommended use concentrations, the QACs and iodophors showed similar resistance to organic matter. At concentrations approved for use on food contact surfaces, both iodophors and QACs are considered superior to hypochlorite with respect to organic matter tolerance. The activity of various QACs compounds on biofilms was studied by Kryszinski et al. (1991). In this study, acid QACs formulations were more effective than neutral or alkaline formulations. Taylor et al. found that at 20°C the effect of 0.3% serum was minimal for several QACs; however, at 10°C, the addition of organic matter reduced activity significantly (Table 16.16).

Effect of Water Hardness

Lack of hard water tolerance has historically been identified as one of the major disadvantages of QACs compared to the halogen-based sanitizers. Hard water tolerance varies significantly with the type of quaternary used (Mosley et al., 1976; Petrocci, 1983), and some products are formulated with a chelating agent (generally EDTA) that negates some of the adverse effects of cations. The type of cations present also seems to influence the degree of interference.

TOXICITY

A comprehensive review of the available literature on the toxicity of QAC was prepared by Petrocci (1983). The author summarized the available toxicologic information as follows:

1. Quaternary ammonium germicides as concentrated solutions of 10% or more are toxic, causing possible death if taken internally and severe irritation to the skin and conjunctival mucosa if applied externally.

TABLE 16.16
Effect of Organic Matter on Antimicrobial Activity of Several QACs at 10°C

Disinfectant Type	Producer Code	Clean/Dirty	<i>Pseudomonas aeruginosa</i> Disinfectant In-Use Concentration			<i>Escherichia coli</i> O157:H7 Disinfectant In-Use Concentration		
			X 0·5	X 1·0	X 2·0	X 0·5	X 1·0	X 2·0
Quat	1	Clean	F	F	P	P	P	P
		Clean	F	F	P	P	P	P
		Dirty	F	F	P	F	P	P
		Dirty	F	F	P	F	P	P
Quat	2	Clean	F	F	F	P	P	P
		Clean	F	F	F	P	P	P
		Dirty	F	F	F	F	P	P
		Dirty	F	F	F	F	P	P
Quat	3	Clean	F	P	P	P	P	P
		Clean	F	P	P	P	P	P
		Dirty	F	P	P	P	P	P
		Dirty	F	P	P	F	P	P
Quat/amphoteric	4	Clean	P	P	P	P	P	P
		Clean	P	P	P	P	P	P
		Dirty	F	P	P	P	P	P
		Dirty	F	P	P	F	P	P
Quat/amphoteric	5	Clean	P	P	P	P	P	P
		Clean	F	P	P	P	P	P
		Dirty	F	P	P	P	P	P
		Dirty	F	P	P	F	P	P

Note: P, Pass; 5 log reduction or greater in viable counts; F, Fail; less than 5 log reduction.

Source: Taylor et al. (1999).

2. With normal precautions and operating procedures, the probability of such contact is extremely remote because effective use concentrations are well below the level causing injury and death.
3. At the normal use concentrations of quaternary germicides (100 to 1000 ppm) only the most deliberate distortions of normal operating procedures could present acute toxicity problems.
4. Chronic toxicity, as from the cumulative effect of food adulterated with quaternary ammonium germicides via residues from dilute sanitizing solutions used on food processing equipment, is not considered a problem.

ACID-ANIONIC SURFACTANTS AND FATTY ACIDS

Anionic surfactants are characterized by a structural balance between a hydrophobic residue (e.g., paraffinic chain or alkyl-substituted benzene or naphthalene ring) and a negatively charged hydrophilic group (e.g., carboxyl, sulfate, sulfonate, or phosphate). The anionics used in approved sanitizing solutions for use on food contact surfaces include dodecylbenzene sulfonic acid, sodium dodecylbenzene sulfonate, sodium dioctylsulfosuccinate, sodium lauryl sulfate, the sodium salt of sulfonated oleic acid, sodium 1-octane sulfonate, sulfonated 9-octadecenoic acid, sodium xylene

sulfonate, dodecylidiphenyloxide disulfonic acid, sulfonated tall oil fatty acid, sodium salt of naphthalene-sulfonic acid, 1-octane sulfonic acid, and a blend of octanoic and decanoic acids (CFR). Fatty acids used as active ingredients in sanitizing solutions include lactic, octanoic nonanoic, and decanoic acids. One of the most comprehensive reviews on acid-anionic surfactants was published by Dychdala (1983b).

MECHANISM OF ACTION

The three most commonly cited hypotheses for mechanism of action of acid amines are as follows: (1) general denaturation of proteins; (2) inactivation of essential enzymes; and (3) disruption of cell membranes, resulting in alterations in permeability. These proposed mechanisms are generally substantiated in several publications (Cornett and Schokman, 1978; Zaslavsky et al., 1978; Yamada, 1979; Dychdala, 1983b)

Fatty acids are active antimicrobials below their pKa. The protonated acids disrupt cell membranes and eventually cause uncoupling of electron transport (Freese and Levin, 1978). In general, fatty acids with a high partition coefficient are the most effective antimicrobial (Kabara, 1978).

ANTIMICROBIAL ACTIVITY

According to Dychdala (1983b), acid-anionic surfactant sanitizers generally possess excellent antimicrobial activity against vegetative Gram-positive and Gram-negative bacteria. Bacterial spores, however, appear to be quite resistant to these compounds. Most yeast strains are inactivated by use dilutions of these compounds, but fungal spores are relatively resistant. Acid-anionics possess virucidal activity against bacteriophage of lactic acid bacteria; however, at normal use concentrations, hypochlorites and iodophors exhibit somewhat superior activity (Hays and Elliker, 1959; Sing et al., 1964a).

Lopes (1986), in his comparative studies with different antimicrobial agents, evaluated the activity of acid-anionic sanitizers against the pathogens *S. Typhimurium* and *L. monocytogenes*. Based on his test results, the recommended use concentrations of acid-anionic sanitizers were effective in controlling both test organisms and compared favorably to chlorine.

Schroeder and Orth (1987) also evaluated acid-anionic surfactant sanitizers (Pennsan CIP) against different species of *L. monocytogenes* isolated by the Pasteur Institute from food processing plants and found that the high concentrations of *Listeria* were effectively killed within 5 minutes, even in the presence of organic contamination such as skim milk (Table 16.17).

Many of the acid-anionic surfactant-type formulations have been replaced by sanitizers using long-chain fatty acids, such as octanoic, and nonanionics. These formulations are low foaming and thus more practical for CIP (clean-in-place) applications.

FACTORS AFFECTING ACTIVITY

Information relating to the effect of various environmental factors on antimicrobial efficacy of acid-anionic surfactants is limited. Again, the reader is referred to Dychdala (1983b) for a general review of this subject. The effect of pH is probably more dramatic than for any other commonly used sanitizers. The bactericidal activity is known to decrease rapidly above pH 3, and a pH range of 1.5 to 3.0 is considered optimal for activity. As the pH is increased above 3.0, the Gram-negative activity is diminished; the Gram-positive sensitivity is still substantial even as the pH approaches neutrality. The effect of acid-anionics in the presence of water hardness components was studied by Dychdala (1959) and the results showed that no slowing effect was observed in the antibacterial activity in water up 1000 ppm hardness (as CaCO₃). The end point of greater than 99.999% reduction with 30 seconds was recorded against *S. aureus* and *E. coli*. However, if the nature of the hardness is such that the pH is increased significantly, beyond 30, the activity is diminished. One might expect that the tolerance to organic material would be similar to that of cationic surfactants (QAC);

TABLE 16.17
Acid-Anionic Composition against *Listeria*^a

Tested Strain	Inoculum (ml ⁻¹)	0.1%	0.2%	0.3%	100 ppm		200 ppm		400 ppm	
					Water	0.1%, 1% Skim Milk	Water	0.25%, 1% Skim Milk	Water	0.5%, 1% Skim Milk
<i>Listeria</i> mhd 78.32 ^b	1.3 × 10 ⁹	0.5	0.5	0.5						
<i>Listeria</i> dgh 78.31 ^b	1.2 × 10 ⁹	0.5	0.5	0.5						
<i>Listeria</i> EHF 78.38 ^b	1.3 × 10 ⁹	0.5	0.5	0.5						
<i>Listeria monocytogenes</i> 1650 ^c	1.9 × 10 ⁹				5	>60	5	5	5	5
<i>Listeria monocytogenes</i> 1293 ^c	9.8 × 10 ⁸				5	>60	5	5	5	5

^a Values given are minutes needed to kill all initially present bacteria at 20°C (68°F) at the given concentrations.

^b Strains isolated from *Listeria monocytogenes* cultures of the Pasteur Institute.

^c Strains are isolated from a cheese brine bath.

Source: Adapted from Schroeder and Orth (1987).

however, published data in this regard are scarce. It is known that the combined effect of low levels of skim milk and water hardness significantly impairs antimicrobial activity. Dychdala (1983b) reported that increases in temperature enhanced bactericidal activity, but decreases from room temperature resulted in slower bactericidal activity. This was confirmed by Taylor et al. (1999).

TOXICITY

An extensive review regarding the acute, subacute, and chronic toxicity of anionic surfactants was published by Potokar (1980). In most cases, LD₅₀ values in excess of 3g/kg have been reported, indicating that acute toxicity is low. Compounds with alkyl chains of 10 to 12 carbon atoms appear to be more toxic than compounds with shorter or longer chain length. Subacute and chronic acute toxicity studies have also been conducted, and none of these surfactants give rise to specific damages. In most cases, the low-grade effects were related to reduced weight gain. Higher dosages produced low-grade changes in the mucous membranes of the intestinal tract, and in some cases, liver and kidney aberrations were observed.

There is some concern regarding the aquatic toxicity of the commonly used anionic surfactants (Gledhill, 1974). In most cases, the more biodegradable linear alkylbenzene sulfonate has replaced the branched-chain tetrapropylene benzene sulfonate.

The toxicity of the anionic surfactants is very low, but use solutions of these sanitizer formulations must be handled carefully because the pH is generally less than 3.0.

AMPHOTERIC SURFACTANTS

The amphoteric surfactants are currently not approved for use in sanitizing solutions in the United States. Because these compounds have been used in Europe for more than 25 years, they deserve at least some mention in this chapter.

These compounds have the general formula R-NHCH₂COOH. Most, if not all, of these compounds are marketed under the trade name Tego. The chemical composition of some of these compounds was described by Block (1983).

MECHANISM OF ACTION

The mode or action for amphoteric surfactants has not been studied extensively. The size of the molecule and its surfactant character lead one to speculate that membrane effects, much like those observed for QAC or anionics, may be involved.

ANTIMICROBIAL ACTIVITY

Manufacturers of amphoteric surfactants claim that their products possess bactericidal, fungicidal, and virucidal activity (Block, 1983). A review of the available literature on these compounds suggests that although bactericidal and fungicidal activity is documented, the contact times required at the recommended use dilutions are longer than for the more commonly used sanitizers (Schmitz, 1952; Sykes, 1965; Block, 1983). The Tego compounds have been shown to adsorb onto solid surfaces and leave a film that resists removal by running water. This property could prove advantageous when residual antimicrobial activity is desirable.

FACTORS AFFECTING ACTIVITY

The effect of various environmental factors on the antimicrobial activity of amphoteric surfactants has not been studied extensively. The pH of the concentrated commercial disinfectants is moderately alkaline. The data presented in Table 16.18 indicate that the antimicrobial activity increases as the

TABLE 16.18
Bactericidal Activities of Amphoteric Surfactants against *Staphylococcus aureus*

	Time for Inactivation (min.)	pH for Optimum Inactivation Rate
Dodecylglycine C ₁₂ H ₂₃ -NH-CH ₂ -COOH	10	3
Dodecylaminoethylglycine C ₁₂ -H ₂₃ -NH-(CH ₂) ₂ -CH ₂ -COOH	5	6–6.5
Dodecyl diaminoethylglycine C ₁₂ H ₂₃ -NH-(CH ₂) ₂ -NH-CH ₂ -COOH	1	9–9.5

Source: Adapted from Schmitz and Harris (1958) in Sykes (1965).

number of nitrogen groups in the surface-active ion increases (Sykes, 1965). This increase in nitrogen groups also brings about a shift in pH for optimum activity (Table 16.18).

The effect of proteins and other organic material on the activity of several Tego compounds was reviewed by Block (1983). The antimicrobial activity of Tego compounds is definitely affected by organic matter. Holah et al. (1990) showed that amphoteric surfactants were effective against bacterial strains under normal growth conditions and produced a 5-log reduction. Against the same organisms in biofilms, however, their activity was much slower and required ten times the initial concentration to produce the equivalent 5-log reduction. Several reports suggest that these compounds may be somewhat less affected by organic matter than other commonly used sanitizers; however, reports that present contradictory data also exist.

TOXICITY

Little information is available on the toxicity of amphoteric surfactants. The manufacturer of the Tego compounds reports that the minimum oral lethal dose of these compounds is 3 g/kg for the undiluted concentrate (Block, 1983). The manufacturer also reports that in liquid soap at a normal concentrate of 1% Tego 51 and 51B, only 0.3% of users showed any evidence of a skin reaction. Based on a limited amount of investigation, the amphoteric surfactants do not appear to present a health hazard under normal use conditions.

PEROXIDES

Peroxides or peroxy compounds are a group of chemical products that contain at least one pair of oxygen atoms (-O-O-) bonded by a single covalent bond. Some of these have distinguished themselves over the years as good antimicrobial agents. Each oxygen has an oxidation number of -1; one of the oxygen atoms is loosely bound in the molecule and is readily released as active oxygen, hence these compounds are referred to as oxidizing agents.

Peroxides may be divided into two basic groups: inorganic and organic peroxy compounds. Inorganic peroxides include hydrogen peroxide (HP), persulfates, perborates, percarbonates, sodium peroxide, and a number of HP addition products. Organic peroxides include peroxyacetic acid (PAA), peroxyoctanoic acid, cumene peroxide, hydroperoxides, diacyl peroxides, peroxyesters, and others. The most widely used peroxy compounds for disinfection and preservation are HP and PAA, both being strong oxidizing agents with varying degrees of antimicrobial activity.

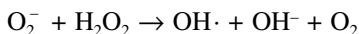
HYDROGEN PEROXIDE

HP was discovered in 1818 by Louis-Jacques Thenard as, what he called, “oxygenated water.” Numerous other scientists did extensive work on “oxygenated water,” or “oxygenated acids.” However, it was the work of B.W. Richardson in 1858 that proposed HP as a disinfectant based on its ability to reduce or remove foul odors. The primary use was as a topical disinfectant at concentrations of 3%. However, because of the relative instability of HP, it met unfavorable results as a topical disinfectant. The manufacturing process used by Thenard persisted until 1950 when an electrochemical process was developed that yielded pure HP in high concentrations that were stable at elevated temperatures and had an extended shelf life (Schumb et al., 1955). This method, also known as organic anthraquinone autoxidation, is still being used today. Currently HP is produced in a stable form at concentrations ranging from 3% to 90%. The stability of HP is dependent on temperature, pH, and the presence of metal ions as well as many other factors.

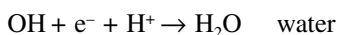
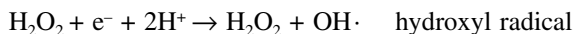
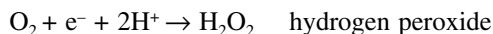
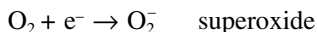
In the past, the use of HP for sanitizing in milk and dairy products was not generally accepted by the regulatory agencies or the dairy industry because of the perceived adulteration of food. There has been a growing tendency to reexamine this viewpoint. A major benefit and a reason for the growth in popularity of HP in the food, cosmetic, and medical fields is its low toxicity at use concentrations as well as its safe decomposition products of water and oxygen. The use of HP in and around food products has been approved in several countries. HP is used in the production of Swiss cheese (Morris et al., 1951) and the treatment of sterile milk (Patterson and Williams, 1952). In cheese manufacturing and milk production, HP is added for microbial control, and the excess is removed by enzymatic reaction with catalase. In more recent years, the FDA has approved the use of HP for sterilization of equipment and containers in aseptic packaging of foods and drinks (CFR, 1990).

MECHANISM OF ACTION

HP is a known natural disinfectant and preservative, present in milk, honey, and mucous membranes of the mouth as well as in neutrophils. Numerous theories on the mechanism of action of HP in the destruction of microbial cells exist. The most prevalent theory is that the formation of hydroxyl radicals, extremely potent oxidants, are primarily responsible for the killing of the microbial cells. The hydroxyl radicals are formed during the reduction of HP.

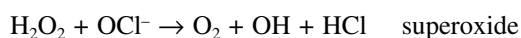
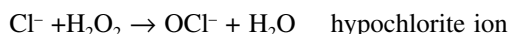


The hydroxyl radical is highly reactive with microbial membrane lipids, DNA, and other cellular components. In 1994, Clapp et al. determined that bacterial activity was inhibited by antioxidants, further implicating hydroxyl radicals as the lethal species. Additional proposed lethal components include the production of superoxides in the reduction series of O_2 (Fridovich, 1975).



The final proposed method involves HP reacting with chloride ions in the cell to produce a hypochlorite ion, a known toxin to microbial cells, and a further reaction of HP and the hypochlorite

to form a superoxide (Klebanoff, 1968). The hypochlorite and superoxide have similar reactions with the microbial cell components as the hydroxyl radical.



Russell (1982) described a mechanism by which the sporicidal activity of HP occurs. He suggested that the sporicidal activity is the result of removing protein from the coat of the bacterial spore.

ANTIMICROBIAL ACTIVITY

HP is effective against a wide spectrum of microorganisms, including bacteria, yeast, molds, viruses and spore-forming organisms. It is also noted that HP activity is greater against Gram-negative bacteria than Gram-positive bacteria. HP inhibits the growth of bacteria at 25 ppm or less and exhibits sporicidal activity at 30,000 ppm within 3 hours at a pH of 5 (Baldry, 1983). However, during the same study, it was noted that HP exhibited poor bactericidal and fungicidal activity. Wardle and Renninger (1975) confirmed good sporicidal activity as well as bactericidal activity within 10 minutes against organisms isolated from spacecraft. Mentel and Schmidt (1973) showed that HP was also a good virucidal agent when tested against rhinovirus.

HP has been used for the disinfection of water (Yoshpe-Purer and Eylan, 1968) and for the sterilization of equipment and containers used in aseptic packaging. It was noted that whereas a contact time of 20 to 30 minutes and a concentration of 30% HP was required to presterilize at room temperature, it only took a few seconds to achieve the same results at 160°F. Toledo (1975) reviewed different sterilants for aseptic packaging applications and concluded that HP at very high temperatures and concentrations will provide the necessary sterilizing effects for these types of surfaces. Rutala (1993) showed that HP at 6% was an effective sterilant within 6 hours. HP has also been used as a vapor-phase sanitizer and sterilant. Klapes and Vesley (1990) demonstrated in their work that vapor-phase HP exhibited a good sporicidal capability and showed promise as a safer alternative to ethylene oxide or formaldehyde.

FACTORS AFFECTING ACTIVITY

Various factors influence the antimicrobial activity of HP such as concentration, pH, temperature, and organic contamination.

Effect of Concentration

As with most antimicrobials, the higher the concentration of HP, the faster the rate of kill of the microorganisms. Concentrations of HP at 0.001% to 0.1% at room temperature are sufficient to inhibit the growth of bacteria and fungi, whereas concentrations of 0.1% and higher are required for bactericidal and fungicidal activity (Table 16.19 and Figure 16.1). Different organisms also show varying resistance to HP when evaluated at the same use concentration. For example, *P. aeruginosa* and *Mycobacterium tuberculosis* are very resistant to HP and considerably higher concentrations are required to achieve comparable kill to other bacteria. Curran et al. (1940) found that by increasing the spore concentration from 1×10^5 to $125 \times 10^5 \text{ ml}^{-1}$, there was an increase in time required to destroy the spores. By doubling the HP concentration from 1% to 2% and 2% to 4%, the destruction time was reduced by approximately 50%.

TABLE 16.19
Percentage Survival of Spores of *B. subtilis* — Exposed Hydrogen Peroxide

H ₂ O ₂ (% concentration)	Exposure Time (min)		
	2	30	60
3	84.8	21.8	2.1
10	34.9	0.0027	0
15	22	0.0022	0

Source: Adapted from Wardle and Renninger (1975).

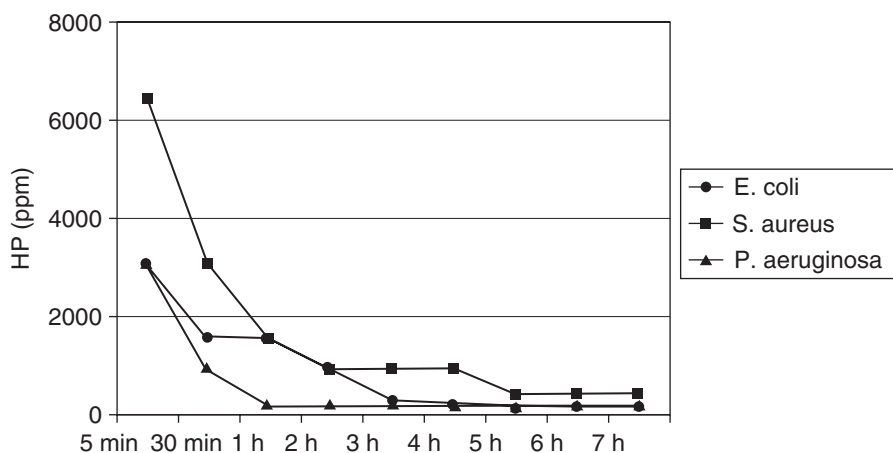


FIGURE 16.1 Minimum bactericidal concentration of hydrogen peroxide against time for water strains. Source: Alasri, A. et al., 1992.

Effect of pH

Changes in pH affect the antimicrobial activity of HP. There appears to be a greater activity of HP in the acid range. This activity remains even in the alkaline pH but to a lesser degree (Table 16.20). Baldry (1983) showed in his study the effect of pH on the inhibitory activity of HP, in which 5 ppm inhibits the growth of *P. aeruginosa* at pH 5, but 10 ppm is required at pH 6.7 and 50 ppm at pH 8. Against spores, a 3% solution of HP killed *B. subtilis* spores in 3 hours at pH 5, but the same solution required 6 hours at pH 6.5 and pH 8.0 (Table 16.20). Although HP shows better antimicrobial activity in acid and slightly lower activity in neutral pH, its overall effectiveness is diminished at alkaline pH.

Effect of Temperature

Temperature has a pronounced effect on the antimicrobial activity of HP. The utilization of higher operating temperatures can result in a lower HP concentration or shorter contact times. High concentrations (3% and higher) and long contact times are required for HP to kill spores. Curran et al. (1940) found that for each 10°C increase in temperature, the destruction of spores increased by one third to one-half using 1% HP. Ito et al. (1973) and Toledo et al. (1973), in their experiments involving bacterial spores using different HP concentrations, different pH levels, and different

TABLE 16.20
Effect of pH on the Sporicidal Activity of H₂O₂ and of PAA

Concentration (ppm)	pH		
	5	6.5	8
H ₂ O ₂			
10,000	>360	>360	>360
20,000	180	360	360
PAA			
830	60	180	>360
8300	<30	<30	<30

Source: Adapted from Baldry (1983).

TABLE 16.21
Disinfection with 3% H₂O₂ Expressed as D Values

Microorganism	D value (min)
<i>Haemophilus influenzae</i>	0.29
<i>Pseudomonas aeruginosa</i>	0.4
<i>Bacillus subtilis</i>	0.5
<i>Escherichia coli</i>	0.57
<i>Proteus vulgaris</i>	0.58
<i>Bacillus cereus</i>	1.04
<i>Proteus mirabilis</i>	1.12
<i>Streptococcus pyogenes</i>	1.5
<i>Staphylococcus epidermidis</i>	1.82
<i>Staphylococcus aureus</i>	2.35
<i>Herpes simplex</i>	2.42
<i>Serratia marcescens</i>	3.86
<i>Candida albicans</i>	3.99
<i>Fusarium solani</i>	4.92
<i>Aspergillus niger</i>	8.55
<i>Candida parapsilosis</i>	18.3

Source: Adapted from Block (2001).

temperatures, came to the following conclusions: (1) under certain use conditions of temperature, concentration, and pH, HP can be sporicidal; (2) as the temperature and HP concentration increases, the sporicidal activity of the solution also increases; (3) survival curves vary in shape depending on temperature, concentration, and the type of organism used; and (4) the relative resistance of spores varies under given conditions of pH, temperature, and HP concentration.

Effect of Other Factors

Other factors affect the antimicrobial activity of HP. Different microorganisms exhibit different resistances to chemicals, and HP is no exception. Turner (1983) showed varying D values for a number of different organisms when tested under identical test conditions (Table 16.21). According to Schumb et al. (1955), the addition of certain inorganic metal ions or salts to HP, such as iron, copper, chromium, and molybdenum, markedly increases the disinfecting power of HP; they

suggested that the formation of free radicals is responsible for this enhanced germicidal activity. Lembke and Teuber (1981) reported that 6% HP exhibited good virucidal activity in aqueous systems, but when evaluated in milk or whey, the inactivation rate of bacteriophages was decreased. This indicates that organic contamination has a slowing effect on the antimicrobial activity of HP. However, Ito et al. (1973) concluded that organic material, free of catalase, had little effect on the sporicidal activity of HP.

TOXICITY

HP is water-like liquid with a characteristically faint acid odor and is usually sold in aqueous solutions at different strengths. The 3% solutions of HP have historically been used for topical skin applications and for wound irrigation in medicinal and cosmetic applications. The 3% solution is not irritating to the skin or eyes. Higher concentrations, such as 5% and higher, are progressively more irritating to skin and eyes. Therefore, great care should be exercised when handling concentrated product to avoid serious injuries. HP at an 8% concentration and higher should be handled with caution because the solution and vapors can cause spontaneous white discoloration on the upper surface of skin and discoloration of mucous surfaces. It may also cause damage to the eyes.

Under no circumstances should HP be taken internally because ingestion may cause injury to the digestive tract and produce internal bleeding. Inhalation of HP vapors or mist may cause extreme irritation and inflammation of the nose, throat, lungs, and other parts of the respiratory tract. HP is not a systemic poison, has low toxicity, and is not a carcinogen or mutagen (Gleason et al., 1969).

PEROXYACETIC ACID

PAA is another strong oxidizing agent that exhibits good antimicrobial activity over a broad range of microorganisms. PAA is more potent than HP, has no harmful decomposition products, is unaffected by catalase and peroxidase, and has germicidal and sterilant properties. Typical commercial formulations that contain PAA (ranging from 4% to 40%) may also contain acetic acid, HP, sulfuric acid, phosphoric acid, and/or a stabilizer. In comparison to HP, PAA is a more potent antimicrobial agent that is effective at cooler temperatures and lower concentrations and is more stable in the presence of organic material (FMC Corp., 1981).

Freer and Novy (1902) first reported the antimicrobial effectiveness of PAA in 1902. They showed excellent bactericidal and fungicidal results using PAA. Later, Hutchings and Xerones (1949) showed in their comparative studies that PAA was superior in activity to 23 antimicrobial compounds they tested. Sproessig and Muecke (1965, 1967) evaluated PAA for use in the medical field and demonstrated bactericidal, sporicidal, and virucidal activity. Because of handling, toxicity, and stability problems, these initial preparations did not gain wide acceptance in disinfection.

In 1975, Grosse-Boewing et al. (1977) developed a stabilized equilibrium mixture. This product retained the biocidal effectiveness of previous compositions based on PAA but eliminated most of the previous stability and handling problems. In 1986, the FDA amended the food additive regulation to include the safe use of PAA in a sanitizing solution on food contact surfaces in food processing plants (CFR, 1990).

The current PAA sanitizers contain 100 to 200 ppm PAA, have little odor, and are stable at room temperature for several days (Figure 16.2). At elevated temperatures the decomposition of PAA is accelerated (Figure 16.3). The concentrated product exhibits good stability if stored under 70°F (Table 16.22).

When diluted, the ingredients of this product decompose into water, oxygen, and acetic acid. These decomposition products are virtually nontoxic when introduced to food or the environment and do not adversely affect wastewater treatment systems under typical use conditions. Other properties include the absence of foam and phosphates, no corrosion to stainless steel or aluminum at use concentration, and good tolerance to hard water.

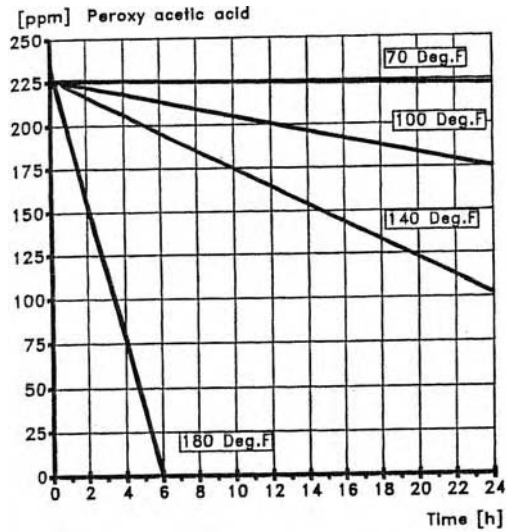


FIGURE 16.2 Stability of peroxyacetic acid (PAA)-based active solutions at 70°F. Adapted from Roshner (1987); Dychdala (1988).

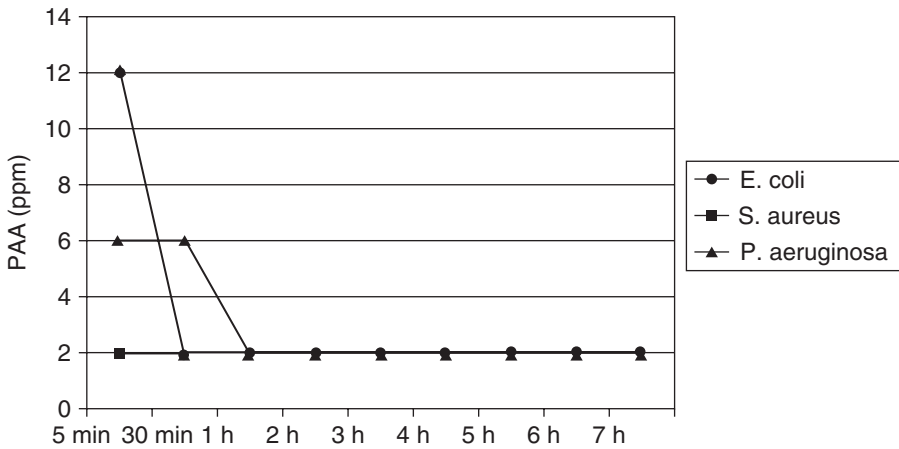


FIGURE 16.3 Temperature stability of 225 ppm peroxyacetic acid (PAA) solution. Adapted from Roshner (1987); Dychdala (1988).

MECHANISM OF ACTION

Similar to the mechanism of action for HP, Clapp et al. (1994) determined that the hydroxyl radicals are the lethal species involved in bactericidal activity. It has been proposed (Shin et al., 1994; Marquis et al., 1995) that sporicidal activity of PAA is increased by reacting with chlorine or iodine present. Vegetative cells can donate electrons from transition metals in the cell membrane to the hydroxyl and superoxide radicals, therefore becoming oxidized and subsequently killed (Rodriguez-Montelongo et al., 1993).

ANTIMICROBIAL ACTIVITY

According to a number of reviews by Schroeder (1984), Block (1986), Roshner (1987), Baldry and Fraser (1988), and Dychdala (1988a,b), PAA is one of the most effective antimicrobial agents

TABLE 16.22
Stability of PAA^a Concentrate under Various
Storage Conditions

Temperature (°F)	Time (months)	Losses of Peroxyacetic (relative%)
60	6	<5
	12	<5
	18	<5
77	6	<5
	12	5
	18	10
95	6	5
	12	10
	18	20

^a 5% PAA

Source: Adapted from Dychdala (1988).

controlling a wide spectrum of microorganisms including bacteria, fungi, viruses, and bacterial spores.

Much of the early microbiological testing of PAA compounds was done in Europe using a variety of microorganisms under different test conditions and product concentrations. Most of the data presented in Tables 16.23 and 16.24 show that PAA is a universally effective antimicrobial agent. These tests were completed using either a suspension test method according to the German Agricultural Association or a suspension and carrier test method according to the German Veterinary Association. Table 16.25 shows that PAA inactivates large populations of *Listeria* at 120 ppm within 1 minute. Similar results were obtained against *Yersinia*, *Campylobacter*, and a variety of viruses (Orth and Mrozek, 1989; Lembke and Teuber, 1981). Holah et al. (1990) evaluated 12 commonly used surface disinfectants using bacterial biofilms developed on stainless steel. The authors concluded that PAA was the most effective of the compounds tested. This was confirmed in biofilm studies by Krysinski et al. (1991) and Carpentier and Cerf (1993) where PAA was compared to quaternary ammonium compounds, chlorine dioxide, chlorine, iodophors, aldehydes, HP, and neutral quaternary ammonium compounds. Fatemi and Frank (1999) showed similar results using mixed culture biofilms. In their study, PAA was more effective than chlorine in the presence of organic challenges (Tables 16.26 and 16.27). Milojkovic (1969) obtained good bactericidal and sporicidal results with a 0.05% to 0.5% PAA solution. At 0.005% concentration, PAA was effective only against vegetative microorganisms, not against spores. Greenspan and MacKellar (1951) demonstrated that PAA solutions were found to be effective against mold growth on tomatoes and that PAA treatment increased the juice yields of the treated tomatoes. Portner and Hoffman (1968) tested the sporicidal activity of PAA vapor at various relative humidity levels and concluded that PAA vapor gave optimal sporicidal activity at 80% relative humidity on both porous and impermeable surfaces.

PAA- and HP-based disinfectants are highly suitable for sanitizing membrane systems because they pass through the membrane easily, are compatible with many membrane materials, and any remaining residue on the equipment does not adversely affect the flavor of the subsequent processed products.

TABLE 16.23
Sporicidal Activity of Peroxyacetic Acid Composition

	Inoculum (ml ⁻¹)	Temperature (°F)	PAA Composition (ppm)		
			200	300	400
<i>Bacillus cereus</i>	3 × 10 ⁷	41	>60 ^a	>60	>60
		50	>60	>60	>60
		68	>60	60	60
		104	40	20	10
<i>Bacillus subtilis</i>	1 × 10 ⁷	41	>60	60	40
		50	>60	>60	40
		68	10	10	5
		104	10	5	1
<i>Bacillus mesentericus</i>	2 × 10 ⁸	41	>60	>60	>60
		50	>60	>60	>60
		68	>60	40	20
		104	60	40	5
Thermophilic spore formers	4 × 10 ⁸	41	>60	40	40
		50	40	40	40
		68	20	5	5
		104	5	2.5	2.5
<i>Clostridium perfringens</i>	1 × 10 ⁷	41	>60	20	10
		50	>60	10	10
		68	20	5	5
		104	2.5	1	1
<i>Clostridium</i> sp.	1 × 10 ⁷	41	40	20	10
		50	40	10	10
		68	2.5	2.5	2.5
		104	2.5	1	1

^a Kill time in minutes (time required to achieve no detectable survivors).

Source: Adapted from Roshner (1987).

FACTORS AFFECTING ACTIVITY

The germicidal activity of PAA is influenced by numerous factors including the concentration, contact time, temperature, and pH of the use solution. Other factors may be organic material contamination and to a lesser extent the effect of hard water.

Effect of Concentration

As with most antimicrobials, increasing the concentration of PAA will yield faster rates of kill as long as pH, temperature, and other factors such as organic contaminants are at a constant. Krzywicka (1970), in her work with spore-forming organisms, showed that *B. cereus* was killed in 3 minutes when exposed to 0.3% PAA, but 90 minutes was required to accomplish the same with a 0.01% concentration of PAA (Table 16.28). Higher concentrations of PAA (up to 0.5%) are required for sporicidal activity in aseptic packaging for the sterilization of equipment and containers where time requirements are limited and sterility is critical.

TABLE 16.24
Antimicrobial Activity of Peroxyacetic Acid Composition

	Inoculum (ml ⁻¹)	Temperature (°F)	PAA Composition (ppm)	
			80	400
Gram-Positive Bacteria				
<i>Staphylococcus aureus</i>	3 × 10 ⁸	41	5 ^a	2.5
		50	2.5	2.5
		68	2.5	1
		104	1	0.5
<i>Streptococcus faecalis</i>	7 × 10 ⁷	41	2.5	2.5
		50	2.5	2.5
		68	2.5	1
		104	1	0.5
Gram-Negative Bacteria				
<i>Pseudomonas aeruginosa</i>	2 × 10 ⁸	41	1	1
		50	2.5	1
		68	1	0.5
		104	1	0.5
<i>Enterobacter aerogenes</i>	4 × 10 ⁸	41	1	1
		50	1	1
		68	1	1
		104	1	0.5
<i>Salmonella</i> Typhimurium	2.4 × 10 ⁹	41	2.5	2.5
		50	2.5	2.5
		68	2.5	2.5
		104	1	1
<i>Salmonella</i> Dublin	1.1 × 10 ⁹	41	2.5	2.5
		50	2.5	2.5
		68	1	1
		104	1	1
Yeast				
<i>Saccharomyces cerevisiae</i>	8 × 10 ⁷	41	20	10
		50	10	5
		68	2.5	1
		104	1	0.5
<i>Saccharomyces diastalicus</i>	9 × 10 ⁷	41	5	2.5
		50	5	2.5
		68	2.5	2.5
		104	1	1
<i>Candida mycoderma</i>	9 × 10 ⁷	41	120	40
		50	90	40
		68	40	10
		104	2.5	1
<i>Hansenula anomala</i>	6 × 10 ⁷	41	>120	40
		50	40	40
		68	10	2.5
		104	1	0.5

(continued)

TABLE 16.24 (CONTINUED)
Antimicrobial Activity of Peroxyacetic Acid Composition

	Inoculum (ml ⁻¹)	Temperature (°F)	PAA Composition (ppm)	
			80	400
<i>Pichia membranaefaciens</i>	9 × 10 ⁷	41	>120	40
		50	60	20
		68	20	10
		104	5	2.5
Molds				
<i>Penicillium cameronense</i>	9 × 10 ⁷	41	>120	90
		50	>120	90
		68	20	10
		104	2.5	1
<i>Aspergillus niger</i>	1 × 10 ⁷	41	>240	>240
		50	>240	>240
		68	90	60
		104	10	5
<i>Mucor</i> sp.	8 × 10 ⁶	41	>240	>240
		50	>240	>240
		68	20	5
		104	2.5	1
<i>Geotrichum candidum</i>	1 × 10 ⁷	41	60	40
		50	40	10
		68	20	5
		104	2.5	1

^a Kill time in minutes (i.e., time required to achieve no detectable survivors).

Source: Adapted from Roshner (1987).

Effect of pH

PAA is optimized as an antimicrobial in an acidic environment. In studies performed by Dychdala and Koroma (1986), it was shown that PAA tested at 0.25% and 0.40% against *E. coli* in 500 ppm hard water exhibited a 99.999% reduction at pH levels of 3.5, 5.5, and 7.0 within 30 seconds. The antimicrobial activity has been shown to decrease with pH ranges above 7, although Tichacek (1966) showed that this may be overcome by significantly increasing the concentration of PAA. Higher pH does not affect the mechanism of activity, but it does slow down the kill of test organisms (Table 16.29). The effect of pH may be a result of the shifting of the equilibrium action of the PAA compounds in a use solution.

Effect of Temperature

PAA exhibits significant bactericidal activity against Gram-positive and Gram-negative organisms even when tested at lower temperatures. Germicidal activity was exhibited against *P. aeruginosa* and *E. coli* at 10°C within 5 minutes, even in the presence of organic load (Taylor et al., 1999). This property gives PAA an advantage over most other types of antimicrobial agents, which are more adversely affected by lower temperatures. Increasing the temperature does show significant improvement in the activity of PAA. This is shown when testing against spore-forming bacteria as well as fungi. Swart (1990) exhibited the effect of temperature on the sporicidal activity of PAA. In this study, sporicidal activity was achieved in 360 minutes at a product concentration of 5% and

TABLE 16.25
PAA Composition against *Listeria*

Strain	Inoculum (ml ⁻¹)	120 ppm	50 ppm		100 ppm		200 ppm	
			Water	1% Skim Milk	Water	1% Skim Milk	Water	1% Skim Milk
<i>Listeria</i> mhd 78.32	1.2 × 10 ⁹	1 ^a						
<i>Listeria</i> dgh 78.31	9 × 10 ⁸	1						
<i>Listeria</i> ehf 78.38	1.9 × 10 ⁹	1						
<i>Listeria monocytogenes</i> 1650	1.9 × 10 ⁹		5	15	1	5	1	5
<i>Listeria monocytogenes</i> 1293	9.8 × 10 ⁸		5	30	1	15	1	5

^a Values represent the time in minutes to kill all initial inoculum at 20°C.

Source: Adapted from Schroeder and Orth (1987).

TABLE 16.26
Survival of 4-Hour Adherent Mixed Cells of *L. monocytogenes* and *Pseudomonas* Species

Microorganism	Sanitizer	Concentration (mg/L) and Exposure Time			
		40		80	
		1 min	5 min	1 min	5 min
<i>Listeria</i>	PAA	4 ^a	3.6	3.8	2.7
	POA/PAA ^b	3.8	4.7	2.9	2.1
	Chlorine	5	3.2	3.6	2.8
<i>Pseudomonas</i>	PAA	4.5	7.2	7.2	3.5
	POA/PAA ^b	3.3	2.7	2	2.2
	Chlorine	7.2	5.2	5.6	4.9

^a Results reported as survivors (CFU/cm²).

^b Mixture of peracetic acid and peroxyoctanoic acid.

Source: Adapted from Fatemi and Frank (1999).

a test temperature of 20°C. The same concentration at 80°C achieved sporicidal activity within 2.5 minutes (Table 16.31). In a study performed by Ecolab (2000b), a 99.999% reduction was exhibited against *A. niger*, *S. cerevisiae*, and *Pediococcus damnosus* within 7 seconds using 380 ppm PAA at 40°C. Jones et al. (1967) compared the activity of PAA and beta-propiolactone against spores at subzero temperatures and showed that 0.3% and 3% solutions were sporicidal over a temperature range of 0°C to -30°C and that PAA was the more active antimicrobial agent. The authors noted that there was an initial lag period that was dependent on the temperature; the lower the temperature, the longer the lag time. At the end of this lag time, the sporicidal activity proceeded rapidly to sterility.

TABLE 16.27
Survival of 4-Hour Adherent Mixed Cells of *L. monocytogenes* and *Pseudomonas* Species in the Presence of a 5% Milk Solution

Microorganism	Sanitizer	Concentration (mg/L) and Exposure Time			
		80		160	
		1 min	5 min	1 min	5 min
<i>Listeria</i>	PAA	5.4 ^a	1.6	1.2	0.7
	POA/PAA ^b	1.3	1.3	0.8	0.5
	Chlorine	TN	TN	>50	>50
<i>Pseudomonas</i>	PAA	4.1	5.4	2.5	1.5
	POA/PAA ^b	4.8	5	2.6	1.8
	Chlorine	TN	TN	>100	>100

Note: TN, survivors too numerous to count.

^a Results reported as survivors (CFU/cm²).

^b Mixture of peracetic acid and peroxyoctanoic acid.

Source: Adapted from Fatemi and Frank (1999).

TABLE 16.28
Effect of Concentration of Peroxyacetic Acid on the Survival of Bacterial Spores (*Bacillus subtilis* ATCC 9372)

Concentration (%)	0.01	0.02	0.03	0.05	0.2
Log reduction	<1	1	2	4	5

Source: Block (2001).

TABLE 16.29
Effect of pH on the Survival of Bacterial Spores (*Bacillus subtilis* ATCC 9372) Exposed to 0.03% Peroxyacetic Acid

pH	2	4	5	7	8
Log reduction	4	3	2	1	<1

Source: Block (2001).

Effect of Other Factors

The antimicrobial activity of PAA does not appear to be greatly affected by water hardness. When tested at 150 ppm PAA against *E. coli* and *S. aureus* in the presence of 500 ppm hard water (using the AOAC germicidal and detergent sanitizing method), a 99.999% reduction was achieved within 30 seconds at room temperature (Ecolab, 2000a). Organic contamination may significantly affect the activity of PAA. The disinfectant effect of PAA on *Mycobacterium bovis* was accomplished

TABLE 16.30
Sporicidal Activity on Peroxyacetic Acid Composition against
***Bacillus subtilis* and *Clostridium sporogenes* versus Temperature**

Test Temperature (°C)	Product Concentration (%)	Exposure Time (min)
20	5	360
20	10	120
50	5	15
80	5	2.5

Note: Exposure time is recorded as time required to achieve sporicidal activity.

Source: Adapted from Swart (1990); unpublished.

TABLE 16.31
Toxicity Data for Peroxyacetic Acid

PAA Concentration	LD50 (mg/kg)	Test Animal	Administration	References
40% solution	1540	Rat	Oral	Lewis and Tatken (1979)
	315	Rat	Oral	Busch and Werner (1974)
	210	Rat	Oral	Merka and Tichacek (1978)
	310	Mouse	Oral	Merka and Tichacek (1978)
	283	Rabbit	Dermal	Phillips and MacPeak (1957)
5% solution	2000	Rat	Oral	HRC Report (1985)
4% solution	3400	Rat	Oral	Roshner (1987)
Ames test for mutagenic effect		Negative		Yamaguchi and Yamashita (1980)
Inhalation lethality, 1000 ppm		Rat, 4-h exposure		Phillips and MacPeak (1957)
Eye irritation, corrosive		Rabbit		Duprat and Gradiski (1974)
Skin irritation, corrosive		Rabbit		Duprat and Gradiski (1974)

Note: PAA, peroxyacetic acid.

Source: Adapted and modified from Kaestner (1981) and Schroeder (1984).

with a 0.0125% concentration within 10 to 20 minutes, but in the presence of sterile bovine feces the concentration of PAA had to be increased to 0.05% to equal the previous activity level (Pavlas, 1967). Although organic contamination does have an effect on the activity of PAA, it is one of the least affected agents when compared to other antimicrobial products. The combined effect of temperature and organic load on 18 different compounds, including peracetic acid, was studied by Taylor et al. (1999).

TOXICITY

Concentrated solutions are potent irritants and powerful oxidizers. Toxicity information is available in literature on the concentrated and the dilute compounds. Table 16.32 summarizes the toxicity information. PAA, depending on the concentration, has a moderate to high oral toxicity. This oral toxicity compares favorably to other antimicrobial compounds on the market. In test conducted on

mice, Merka and Tichacek (1976) reported negative results on test animals when exposed to the maximum level of PAA in air (70 mg/m³). These tests also showed that repeated exposure to PAA induced temporary morphologic lesions in the lungs; however, a rapid regeneration of tissues occurs when exposure to PAA is removed.

In inhalation studies, 5% PAA compounds and the undiluted product were sprayed into a chamber containing rats for a duration of 4 hours. The results showed that rats treated with a 5% solution did not exhibit any toxic symptoms during or after the tests. Those treated with the concentrated product showed symptoms of discomfort that disappeared after termination of the test (Kaestner and Gloxhuber, 1977). These authors reported in a separate study with human volunteers that a 3% solution of active P3-oxonia applied to the lower arm for a period of 30 minutes resulted in no visible skin reaction during or after the treatment. In another experiment, a 5% solution of PAA compound was applied twice a day for 2 weeks to a quarter-sized area of skin on the backs of 10 hairless mice. A slight initial redness of the skin was observed on only 2 or 3 test animals after treatment 4 or 6. After further consecutive applications with PAA, none of the remaining animals exhibited any symptoms.

At higher concentrations, PAA is a strong skin and mucous membrane irritant and on repeated contact has a corrosive effect on skin and mucous surfaces. In tests with laboratory animals, PAA is corrosive to the eyes and causes irreversible damage. Should PAA come into contact with eyes or skin, the area should be immediately flushed with plenty of water and prompt medical attention should be sought.

Kaestner (1981) tested long-term feeding studies when drinking water was disinfected using a 0.02% PAA concentration. This water was fed to rats, mice, hamsters, gerbils, and guinea pigs for a period of 10 months. After these studies, no abnormal effects were observed on any of the test animals. Histologic examination also did not show any abnormalities in the inner organs.

Kaestner (1981) reported that Sproessig et al. (1978), in their independent studies, treated the skin of mice for 1 year at regular intervals with 0.2% to 2% PAA solutions without observing any carcinogenic or tumor-promoting activity. The EPA Cancer Assessment Group and the International Agency for Research on Cancer does not list PAA as a carcinogen. Using the Ames test, Yamaguchi and Yamashita (1980) evaluated the mutagenicity of peroxygen compounds and reported that PAA was not mutagenic.

When handling PAA products, extreme care must be exercised to prevent direct contact with the product by wearing protective clothing, safety goggles, face shield, rubber gloves, and rubber boots. Skin and eye protection is absolutely essential when handling more concentrated products, in accordance with instructions for shipping and handling. If for any reason an accidental contact with PAA occurs on skin, prompt rinsing with water not only dilutes the product but also should prevent or minimize the irritation, as well as other toxicity risks.

SUMMARY

REGULATORY REQUIREMENTS

It is required that manufacturers of sanitizing solutions who claim antimicrobial activity for their formulation register the formulation with the EPA. The proposed rules for these registrations and the required test methodologies are published in the *Federal Register* (EPA, 1989).

The FDA has published a list of approved sanitizing solutions that do not require a potable water rinse (CFR, 1990). In certain cases, the U.S. Department of Agriculture (USDA) does permit the use of sanitizers at higher levels than permitted by the FDA. Chemicals listed as generally recognized as safe (GRAS) or permitted for use by prior sanction or by food additives approval may also be used in sanitizing solutions. This permits the use of gaseous chlorine, edible organic acids, and strong alkali.

TABLE 16.32
Comparison of the Commonly Used Sanitizers in Antimicrobial Activity

Cidal Activity against	Chlorine	Iodophors	Quaternary Ammonium Compounds	Acid Anionic Surfactants	Peroxyacetic Acid
Gram-positive Nonspore-forming bacteria	++	++	++	++	++
Gram-negative bacteria	++	++	+	+	++
Bacterial spores	++	+	±	±	++
Yeast	+	++	+	+	+
Mold	++	++	+	+	++
Bacteriophage	++	+	±	±	++

Note: ++, effective; +, moderately effective; ±, variable effectiveness.

PROPERTIES FOR APPROVED SANITIZERS

It is difficult to make direct comparisons between the various types of sanitizing solutions because of the variability in formulations among the commercially available products. The information provided in Tables 16.32 and 16.33 is based on the most commonly used commercial products. Exceptions to the norm may be encountered, especially with respect to iodophor and quaternary ammonium compounds. The manufacturers of these products can provide the user with information relating to antimicrobial efficacy and other factors relevant to the use of specific products.

ANTIMICROBIAL TREATMENTS APPLIED TO FOOD TISSUE SURFACES

RED MEATS AND POULTRY

Carcass washing has become an integral step in the pathogen reduction program of many slaughter facilities. The integration of antimicrobials into this step has increased food safety. The USDA has approved several antimicrobial sprays that can be used in meat processing facilities. They are as follows: the use of a water rinse followed by a second rinse with an organic acid; a chlorinated water (20 to 50 ppm) wash; GRAS organic acids sprays at 1.5% to 2.5%; and food-grade trisodium phosphate sprays of 8% to 12% (Pordesimo et al., 2001). The washing of carcasses has progressed from simple handheld sprayers to large stainless steel cabinets with multiple oscillating spray nozzles to ensure that the entire carcass is adequately covered with the solution. This intervention step has been evaluated based on application; for example, Castillo et al. (1999) evaluated the benefits of spraying carcasses after they had been in the chiller for extended periods. Further studies have been performed to distinguish the importance of such variables as temperature, pressure, concentration, spray time, and combinations of multiple spray treatments. The following section addresses some of the work that has been performed on the more common antimicrobials used as carcass treatments.

RED MEATS

Chlorinated Compounds

Sodium hypochlorite alone has proved to be ineffective as a red meat carcass treatment because of the high amount of organic matter on the surface of the carcass (Castillo et al., 1999). The high organic load reduces the bactericidal activity of hypochlorous acid by reducing the amount of available chlorine. The process of combining an organic acid with sodium chlorite to create chlorous acid and chlorine dioxide, depending on the pH of the solution, can overcome this reduction in bactericidal activity. Acidified sodium chlorite has shown promising results as an antimicrobial for red meat surfaces. The work done by Castillo et al. (1999) showed that acidified sodium chlorite adjusted to a final chlorous acid concentration of 164 mg/L was more effective than water spray alone in reducing the numbers of *E. coli* O157:H7 and *S. Typhimurium* on warm boned beef carcass regions. Their data showed \log_{10} reductions of 2.2 and 2.3 for *E. coli* and *S. Typhimurium*, respectively, when compared to a water spray.

Organic Acids

The use of organic acid carcass sprays has been studied extensively. Researchers have drawn different conclusions regarding the efficacy of organic acids depending on the variables that existed during treatment. Some of the factors that have contributed to the variation in results are type of meat (e.g., lean, adipose, connective), temperature of spray, pressure of spray, concentration of organic acid, type of organic acid, storage temperature after treatment, and whether the carcass was chilled before treatment. According to Cutter and Siragusa (1994), the type of organic acid is not as significant as the type of tissue being treated and the concentration of the organic acid being used. In their work, concentrations of 1%, 3%, and 5% acetic, lactic, and citric acids were sprayed on lean and adipose beef regions inoculated with *E. coli* and *Pseudomonas fluorescens*. The greatest reductions were seen on adipose tissue sprayed with 5% of any organic acid. Their results show that the effect of adipose versus lean tissue had a greater influence on results than did the concentration at which the organic acid was applied. Differences in reductions of *E. coli* and *P. fluorescens* between adipose and lean tissue were approximately 1 and 2 \log_{10} CFU g^{-1} , respectively. A possible explanation for the greater reductions when treating adipose tissue is that the bacterial cells will be less firmly attached when compared with lean red meat tissue.

Hardin et al. (1995) performed similar work using a prewash step of water tempered at 35°C followed by a spray with either 2% lactic or 2% acetic acid at 55°C. Their results showed 2% lactic acid was more effective in reducing populations of *E. coli* O157:H7 on the inside round, outside round, brisket, and clod than 2% acetic acid. Likewise, greater reductions were seen when beef carcass regions contaminated with *S. Typhimurium* were prewashed with water followed by a 2% lactic acid spray than when sprayed with 2% acetic acid following a water prewash. Overall, the results of Hardin et al. (1995) showed red meat prewashed with water followed by an organic acid antimicrobial spray is more effective than a water wash alone. Also, lactic acid proved to be more efficacious to both organisms tested having approximately 1.3 \log_{10} better reductions on all tissue types. Furthermore, Hardin et al. (1995) observed the greatest reduction of organisms tested on the outside round, which inherently contains more adipose than the other tissue types. This data supports earlier findings that greater reductions are seen in adipose tissue treated with organic acids than lean tissue.

Hydrogen Peroxide

A concern regarding the use of HP as a carcass treatment is that the residual HP may whiten meat tissue. The work performed by Bell et al. (1997) showed that tissue treated with HP at near neutral pH had very little residual HP versus tissue treated with HP at a lower pH. Bell et al. (1997)

TABLE 16.33
Comparison of the Commonly Used Sanitizers in Chemical and Physical Properties

	Chlorine	Iodophors	Quaternary Ammonium Compounds	Acid Anionic Surfactants	Peroxyacetic Acid
Corrosiveness	Corrosive	Slightly corrosive	Noncorrosive	Slightly corrosive	Slightly corrosive
Irritating to skin	Yes	No	Noncorrosive	Slightly irritating	No
Effective at neutral pH	Yes	Dependent on type	Most cases	No	Yes
Effective at acid pH	Yes, unstable	Yes	Some cases	Yes, below 3.5	Yes
Effective at alkaline pH	Yes, less than neutral pH	No	Most cases	No	Less effect
Affected by organic material	Yes	Moderately	Moderately	Moderately	Partially
Affected by water hardness	No	Slightly	Yes	Slightly	Slightly
Residual antimicrobial activity	None	Moderately	Yes	Yes	None
Cost	Low	High	Moderate	Moderate	Moderate
Incompatibilities	Acid solutions, phenols, amines	High alkaline detergents	Anionic wetting agents, soaps, and acids	Cationic surfactants and alkaline detergents	Reducing metal ions, alkalis
Stability of use solution	Dissipates Rapidly	Dissipates Slowly	Stable	Stable	Dissipates Slowly
Maximum level permitted	200 ppm	25 ppm	200 ppm	430 ppm dodecylbenzene sulfonate, 200 ppm sodium salt of oleic acid, 350 ppm C8–C10 fatty acids	100–200 ppm
Water temperature sensitivity	None	High	Moderate	Moderate	None
Foam level	None	Low	Moderate	Low	None
Phosphate	None	High	None	High	None
Soil load tolerance	None	Low	High	Low	Low

investigated the use of HP alone and in conjunction with organic acids and sodium bicarbonate as surface sanitization sprays for both lean and adipose red meat tissues (Table 16.35). They found that a combination of 1% acetic acid and 3% HP provided the greatest reduction of *E. coli*, *Listeria innocua*, and *Salmonella* Wentworth. The authors suggest a possible explanation for the increased reduction may be the result of a synergistic effect of organic acid and HP. This combination of antimicrobials may have a similar mechanism of action to PAA. However, the decrease in surface pH of the carcass leads to an increase in residual HP. Similarly, the work done by Delazari et al.

TABLE 16.34
The Average Log₁₀ Reduction (CFU cm⁻²) of *Escherichia coli*, *Listeria innocua*, and *Salmonella* Wentworth on Two Red Meat Tissue Types

Organism	Treatment ^a	Adipose Tissue	Lean Tissue
<i>Escherichia coli</i>	3% hydrogen peroxide	2.71	3.34
	1% acetic acid/3% hydrogen peroxide	2.92	3.92
	1% sodium bicarbonate/3% hydrogen peroxide	3.15	2.76
<i>Listeria innocua</i>	3% hydrogen peroxide	2.95	3.39
	1% acetic acid/3% hydrogen peroxide	3.05	3.79
	1% sodium bicarbonate/3% hydrogen peroxide	3.34	3.18
<i>Salmonella</i> Wentworth	3% hydrogen peroxide	2.66	3.51
	1% acetic acid/3% hydrogen peroxide	3.83	3.65
	1% sodium bicarbonate/3% hydrogen peroxide	2.57	2.60

^a Spray treatments at 80 psi for 15 seconds at 25°C.

Source: Adapted from Bell et al. (1997).

(1998) showed that a prewash step with 74°C water followed by a 5% HP spray reduced *E. coli* populations on beef carcasses by 3.02 log₁₀.

Peroxyacids

Surface treatment of red meat with a mixed peroxyacid spray has yielded promising results as an effective antimicrobial used to reduce populations of microorganisms on beef carcasses. Like HP, PAA application leaves benign residuals on meat (acetic acid, water, oxygen). The advantage of using peroxyacid sprays instead of HP is that the peroxyacid can be used at low enough concentrations to prevent discoloration of the meat and still achieve significant reductions in bacteria attached to the meat surface. Gutzmann et al. (2000) have shown that the effectiveness of peroxyacid sprays is dependent on the pressure of spray, the concentration of the peroxyacid, and the temperature at which it is applied (Table 16.35).

TABLE 16.35
Average Log₁₀ CFU mL⁻¹ Reduction of Prerigor Beef Bacterial Flora Using a Combination of Mixed Peroxyacid Spray Treatments

Mixed Peroxyacid Concentration (ppm)	Temperature of Spray (°C)	Pressure of Spray (psi)	Spray Time (sec)	Log ₁₀ Reduction
50	38	230	30	0.38
100	38	230	30	2.22
200	32	50	10	0.41
200	38	65	30	0.38
200	38	230	10	0.96
200	38	230	30	>2.90
200	49	50	10	0.97
500	37	25	30	0.93
500	49	25	10	0.67

Source: From Gutzmann et al. (1999).

Trisodium Phosphate

Trisodium phosphate (TSP) at concentrations to 12% is approved for spraying on red meat carcasses at temperatures up to 44°C for no longer than 30 seconds. The work done by Dorsa et al. (1998) details the effectiveness of TSP sprayed in a wash cabinet for 15 seconds at 80 lb/in² and 32 ± 2°C as a red meat carcass spray. Their results indicate that TSP used as a surface treatment for beef when compared with water treatment alone did not show significant differences in the least square mean counts for an assortment of different organisms.

Ozone

Ozone is GRAS for bottle water rinses within the United States and has been approved as a secondary direct food additive by the FDA. Ozone has proved to be a versatile antimicrobial showing promising results both as a gas and in aqueous solution; its mode of action for inactivating the bacterial cell is the same in both forms. Ozone is an unstable oxygen allotrope and is a strong oxidizer that targets microbial cell nucleic acid (Kim et al., 1999). However, the effectiveness of ozone in reducing populations of pathogenic bacteria is decreased as it is applied to the surface of carcasses compared to its effectiveness in a low ozone-demand liquid media (Kim et al., 1999). According to a review by Kim et al. (1999), rinsing beef carcasses with ozonated water at concentrations of 0.3 to 2.3 ppm reduces total aerobic plate counts by 1.3 log₁₀ CFU cm⁻². However, other researchers have shown that beef surfaces treated with 100 ppm gaseous ozone for 30 minutes had little or no effect on microbial contamination and discolored the meat (Kim et al., 1999).

POULTRY

Chlorinated Compounds

Chlorine in the form of hypochlorous acid is widely used in carcass hydrochilling systems by poultry processors. The benefits of using chlorine are its low cost, established efficacy, and wide availability (Tsai et al., 1992). The major drawback of using chlorine in chiller tanks is its loss of effectiveness as organic matter increases in the chiller water during production. To compensate for this, additional hypochlorite must be added to the chiller water in relatively high concentrations or dose intervals. However, excessive chlorination of food processing water with hypochlorite has prompted concern over production of harmful organochlorine compounds. Chloroform, N-chloro compounds, chlorinated purine and pyrimidines, chlorophenols, and chlorobenzenes are some of the carcinogenic and toxic compounds resulting from chlorination of food products and water (Cheng-I et al., 1985; Fukayama et al., 1986). Furthermore, it has been shown that the high levels of chlorine needed to compensate for the organic load can alter meat taste and cause carcass discoloration (Tamblyn and Conner, 1997).

Another chlorinated compound used in poultry processing facilities to reduce surface contamination on poultry carcasses is ASC spray. Acidified sodium chlorite is typically prepared by using citric acid to acidify sodium chlorite creating hypochlorous acid and chlorine dioxide, depending on the pH of the solution, which are broad-spectrum antimicrobials. The results of a study performed by Kemp et al. (2000), compared the reduction of bacteria obtained from treating poultry carcasses with different concentrations of ASC applied as a dip or a spray (Table 16.36). Their results show for all organisms tested that dipping the carcasses in 1200 ppm acidified sodium chlorite was more effective than spraying the carcasses with the same concentration of acidified sodium chlorite. The authors were able to show that carcasses exposed to 1200 ppm ASC for 5 seconds had a greater log₁₀ reduction in microbial contamination than carcasses exposed to 20 ppm chlorinated water for 1 hour. Kemp et al. (2001) also investigated the use of ASC spray treatment to follow an inside-outside-bird-washer (IOBW) in order to eliminate the need for manual/off-line reprocessing of visually contaminated carcasses. These data show significantly greater reductions in the number of

TABLE 16.36
Average \log_{10} Reductions in Bacteria Obtained from Chicken Skin Treated with Different Antimicrobials

Antimicrobial Agent	Concentration	Application	\log_{10} Reduction	Reference
Mixed peroxyacid	200 ± 10 ppm	40–60 psi spray	3.24 (CFU mL ⁻¹)	Food Additive Petition FAP 1A4728 filed by Ecolab, Inc. (2001)
Trisodium phosphate	8%	15-min dip	1.69 (CFU g ⁻¹)	Capita et al. (2001)
	10%		2.08 (CFU g ⁻¹)	Capita et al. (2001)
	12%		2.51 (CFU g ⁻¹)	Capita et al. (2001)
Mixed peroxyacid	200 ± 10 ppm	40–60 psi spray	2.01 (CFU mL ⁻¹)	Food Additive Petition FAP 1A4728 filed by Ecolab, Inc. (2001)
Cetylpyridinium chloride	0.1%	15°C spray	0.87 (CFU cm ⁻²)	Kim and Slavik (1996)
	0.1%	50°C spray	1.70 (CFU cm ⁻²)	Kim and Slavik (1996)
	0.1%	3-min dip	1.52 (CFU cm ⁻²)	Kim and Slavik (1996)
Mixed peroxyacid	200 ± 10 ppm	40–60 psi spray	3.27 (CFU mL ⁻¹)	Food Additive Petition FAP 1A4728 filed by Ecolab, Inc. (2001)
Acidified sodium chlorite	1200 ppm	5-sec dip	1.23 (CFU mL ⁻¹)	Kemp et al. (2000)
		15-sec spray	0.77 (CFU mL ⁻¹)	Kemp et al. (2000)
Mixed peroxyacid	200 ± 10 ppm	40–60 psi spray	3.81 (CFU mL ⁻¹)	Food Additive Petition FAP 1A4728 filed by Ecolab, Inc. (2001)

pathogenic bacteria removed by the online acidified sodium chlorite reprocessing spray than the manual offline reprocessing method. The average \log_{10} CFU mL⁻¹ recovery of *E. coli* in rinsate from poultry carcasses treated with the online ASC system (IOBW plus ASC spray) was 0.59, whereas the average \log_{10} CFU mL⁻¹ recovery of *E. coli* from poultry carcasses that were cleaned using off line reprocessing was 2.37.

Organic Acids

The use of organic acids as antimicrobials during the chilling of poultry carcasses has been shown to reduce the amount of bacteria in the chiller water, which in turn reduces cross-contamination. However, *Salmonella* cells that are attached to the surface of the skin are normally unaffected by this treatment (Tamblyn and Conner, 1997). The authors evaluated the use of organic acid in a chicken scald to reduce the numbers of *S. Typhimurium* attached to the skin of chicken carcasses. Their results indicate that organic acids are more effective in reducing *S. Typhimurium* attached to the skin of chicken carcasses as both the application temperature and concentration of the acid are increased. They also demonstrated a noticeable difference in the \log_{10} reduction of *Salmonella* cells depending on which organic acid is used. Lactic acid at 1.0% outperformed all of the other acids tested at 6.0%.

Hydrogen Peroxide

According to Dickens and Whittmore (1997), the plate counts of chicken carcasses sprayed during the defeathering process with water and with 0.5%, 1.0%, and 1.5% HP were not significantly different.

TABLE 16.37
Log₁₀ CFU mL⁻¹ Recovery of *Escherichia coli*
from Chiller Water with Different Treatment

Treatment	Log ₁₀ Recovery
Before chiller	1.51
Chlorine chill (2 ppm)	0.82
Mixed peroxyacid chill (25–40 ppm)	0.11

Source: Unpublished data.

TABLE 16.38
Reductions (Log₁₀ CFU cm⁻²) of Microorganisms on Apples, Lettuce,
and Tomatoes following Treatment with 200 ppm Chlorine for 1 min

Microorganism(s)	Apples	Lettuce	Tomatoes
<i>E. coli</i> O157:H7	1.14	3.56	2.76
<i>L. monocytogenes</i>	0.80	ND ^a	0.33
Aerobic mesophiles	2.35	3.92	1.57
Yeasts and molds	2.72	ND	1.91

^a Not determined.

Source: Beuchat et al. (1998).

Peroxyacids

Using a combination of several peroxyacids as a poultry carcass spray as well as in chiller tanks has shown promising results in reducing pathogens on poultry carcasses. Data submitted by Ecolab, Inc., in a petition for secondary direct food additive status for a mixed peroxyacid system, shows significantly greater reductions than a water spray (Table 16.37). Additional data generated showed that a combination of peroxyacid spray with a low-concentration chiller application provided even greater reductions than a peroxyacid spray alone. The combination of a carcass spray with 200 ± 10 ppm total peroxyacid at 50 to 55 psi and a 25 to 40 ppm total peroxyacid chill showed *E. coli* reductions of 1.44 log₁₀ in pilot plant tests (unpublished data). Chicken carcasses treated with a mixed peroxyacid solution at concentrations of 25 to 40 ppm in the chiller water alone showed significantly lower recovery of *E. coli* than carcasses chilled in water containing 2 ppm free chlorine (Table 16.38).

Trisodium Phosphate

TSP has shown good results for use as a poultry carcass spray and hydrochiller treatment, although the latter application is not commercially practiced. Capita et al. (2001) evaluated the use of TSP as an additive to the chiller water to reduce *L. monocytogenes* on the chicken skin. Their work compared the differences in bacterial recovery from chicken skins after being treated in a hydrochiller with different concentrations of TSP or with potable water alone (Table 16.36). Results of their work show log₁₀ CFU g⁻¹ reductions of *L. monocytogenes* ranging from 1.69 to 2.51 when compared to potable water treatments. Similar work with TSP spray applications has been done by Xiong et al. (1998). They investigated the use of 5% and 10% TSP prechiller sprays as a possible

way to reduce *S. Typhimurium* cells attached to chicken skin. The skin was sprayed at room temperature for 30 seconds at 207 kPa. Following recovery of the surviving *S. Typhimurium*, the 5% and 10% spray solutions had \log_{10} reductions of 2.1 and 2.2, respectively; whereas treatment with a water spray resulted in a \log_{10} reduction of 1.3.

Ozone

Ozone has been evaluated for use as a carcass wash and for use in chiller water. According to a review written by Kim et al. (1999), researchers have found that the total microbial counts on chicken carcasses treated with chiller water that contained 3.0 to 4.5 ppm ozone were repeatedly lower than those carcasses treated with tap water alone. Similarly, work done by Sheldon and Brown (1986) indicates poultry carcasses rinsed with ozonated water exhibited a decrease in the recovery of the total bacterial load. The \log_{10} recovery of organisms from carcasses treated with potable water alone was 5.95, and the \log_{10} recovery from carcasses treated with ozonated water was 5.56.

Cetylpyridinium Chloride

Cetylpyridinium chloride (CPC), a quaternary ammonium compound commonly found in oral hygiene products, has been investigated as a poultry carcass treatment. CPC appears to have rapid bactericidal activity, according to Kim and Slavik (1996). CPC at a concentration of 10 ppm can achieve a 6 \log_{10} reduction of *S. Typhimurium* in liquid suspension in 1 minute. The mode of action of CPC is similar to other quaternary ammonium in that it forms ionized compounds preventing bacterial metabolism, thereby destroying the cell (Kim and Slavik, 1996).

Kim and Slavik (1996) investigated the use of CPC on poultry carcasses either as a spray treatment or as an antimicrobial dip to reduce the contamination of *S. Typhimurium*. Spray treatments were performed with 0.1% CPC solutions at 15°C or 50°C for 1 minute at 138 kPa with potable water sprays performed as controls. The chicken skin that was evaluated for the dipping procedure was exposed to 2.5 mL of 0.1% CPC or potable water and held for 3 minutes before rinsing. Their results show the greatest \log_{10} reduction of *S. Typhimurium* was obtained with a 50°C 0.1% CPC spray. Similar work was done by Breen et al. (1997). However, their work compared the reductions of *S. Typhimurium* based on the concentrations of CPC used as well as the time the skin was exposed to the CPC solution. Exposure time was directly proportional to the reduction of viable *S. Typhimurium* cells.

RAW FRUITS AND VEGETABLES

In recent years, there has been a marked increase in the number of documented outbreaks of human illness associated with the consumption of raw fruits and vegetables in the United States and Canada (Tauxe et al., 1997; Beuchat, 1996; De Roever, 1998). Concomitantly, increases in the size of producers, global distribution, as well as the consumption of raw produce have also been observed, which may contribute to the changing epidemiology of produce-related foodborne disease (Beuchat, 1996; Brackett, 1999).

Gram-negative bacteria dominate the microflora associated with most vegetables, whereas mold and weakly fermentative yeasts often comprise the majority of the microflora on raw fruits because of the acidic pH of the fruit tissue, which is generally less than 4.0 (Burnett and Beuchat, 2000). The majority of the microorganisms associated with produce tissues contribute to product spoilage. Although human pathogens are sporadically isolated from fruits and vegetables, their presence is cause for public health concern (Brackett, 1992).

The use of antimicrobials in the produce industry has traditionally been associated with the control of cross-contamination in flume waters used to remove soil from fruits and vegetables. Chemical treatments, however, have not been demonstrated to reduce populations associated with

the surfaces and tissues of produce by more than about 3 log₁₀ (Beuchat et al., 1998). Limitations in effectiveness have been attributed to the inability of the potentially lethal chemical components to access microorganisms within discontinuities of the surface and tissues of produce (Adams et al., 1998; Seo and Frank, 1999; Burnett and Beuchat, 2002). Treatment with sanitizers, however, is a critical component in the process of providing wholesome and safe produce to the consumer. Summarized here are several chemical treatments that have been applied to raw produce.

Chlorinated Compounds

Chlorine has been used for many years as a sanitizer in wash, spray, and flume waters in the fresh produce industry. Its effectiveness to reduce microbial populations on several types of produce, and under several test parameters (e.g., temperature, exposure time, etc.) has been investigated. To sanitize produce, concentrations of 50 to 200 ppm free chlorine are typically used, with a contact time of 1 to 2 minutes (Beuchat, 1998).

As with other chemical sanitizing treatments, the efficacy of chlorine in killing microorganisms associated with produce is probably most influenced by the surface topography of the fruit or vegetable being sanitized. In their study to determine the influence of chlorine treatment on populations of pathogenic bacteria and resident microflora, Beuchat et al. (1998) demonstrated a significant influence of efficacy by the type of produce undergoing treatment (Table 16.38). Treatment of shredded lettuce and diced tomato with 200 ppm chlorine has been determined to differ in terms of reductions of *Salmonella* by over 0.5 log₁₀ between the two produce types (Weissinger et al., 2000).

Inactivation of free chlorine as a result of contact with the surfaces and tissues of produce, as well as with soil and other field debris, is an important limiting factor in the overall effectiveness of hypochlorite. Careful monitoring of the level of free chlorine, in addition to pH, is paramount in chlorinated waters used in the industry. In a study to determine the microbial changes in fresh market tomatoes during packing operations, Senter et al. (1985) observed that total plate counts and populations of Enterobacteriaceae were higher, compared to controls, on tomatoes washed in water containing an average of 114 ppm chlorine. However, decreases were noted, compared to controls, when tomatoes were treated in water containing 226 ppm chlorine.

The effects of the concentration of free chlorine on microorganisms associated with raw produce have been investigated. Populations of pathogens on leafy salad greens were markedly reduced with increasing concentrations of chlorine to 50 ppm, but no substantial effect was observed by increasing the concentration to 200 ppm (Mazollier, 1988). Similarly, Zhuang et al. (1995) determined that increasing the concentration of chlorine to 320 ppm from 110 ppm had no significant influence on populations of *Salmonella* Montevideo on green tomatoes following treatment. However, the use of 2000 ppm chlorine has been recommended for the treatment of seeds for sprouting, following studies that demonstrated the inability of lower concentrations to effectively kill pathogenic bacteria on seed surfaces (Jaquette et al., 1996; Taormina and Beuchat, 1999).

Chlorine dioxide (ClO₂) has received attention in recent years as an alternative sanitizer to chlorine in the fresh produce industry. Aqueous ClO₂ as a sanitizer can be used as an antimicrobial agent in water used to wash fruits and vegetables in an amount not to exceed 3 ppm residual ClO₂. Gaseous ClO₂ has also garnered growing attention among scientists to determine its effectiveness as a sanitizer for produce.

Compared to the information that is available on chlorine and produce sanitization, little can be found related to ClO₂. Aqueous ClO₂ was evaluated and found to be effective against conidia and sporangiospores of several fungal pathogens of apples (Roberts and Reymond, 1994). Reductions of >99% in viable spores were observed within 1 minute of exposure to 3 or 5 ppm ClO₂. Treatment of shredded lettuce with 3 ppm ClO₂ for up to 10 minutes resulted in a reduction of only 0.7 log₁₀ CFU/g of *L. monocytogenes* (Zhang and Farber, 1996). Minor reductions of bacteria associated with produce surfaces by aqueous ClO₂ were substantiated by Reina et al. (1995).

Although they determined that ClO_2 at a concentration of 1.3 ppm optimally controlled aerobes and Enterobacteriaceae suspended in chiller water used for hydrocooling pickling cucumbers, bacterial populations on or in cucumbers were not greatly influenced by chlorine dioxide, even at 5.1 ppm. The authors suggested that microorganisms on or in the fruit were protected from ClO_2 .

The ability of gaseous ClO_2 to penetrate protective sites on produce to kill microorganisms was demonstrated by Yan et al. (2000a, 2000b). Treatment of green peppers inoculated with *E. coli* O157:H7 with 0.62 or 1.24 ppm ClO_2 resulted in reductions of 3.0 and 6.4 \log_{10} , respectively.

ASC has been approved for use as an antimicrobial agent on raw produce applied as a dip or spray at concentrations of 500 to 1200 ppm. Information regarding its effectiveness to decontaminate produce is limited in the literature. ASC was used to treat cantaloupes, honeydew melons, and asparagus inoculated with *E. coli* O157:H7 and *Salmonella* (Park and Beuchat, 1999). Reductions of 3.38 and 2.65 \log_{10} in populations of *E. coli* O157:H7 and *Salmonella*, respectively, were observed. It was concluded that treatment with 850 ppm ASC was as effective as treatment with 2000 ppm chlorine.

Hydrogen Peroxide

The efficacy of HP in improving the microbiological quality and extending the shelf life of raw and minimally processed produce has been studied extensively by Sapers and coworkers (1999, 2000, 2001a, 2001b, 2003; Ukuku and Sapers, 2001; Annous and Sapers, 2001; Liao and Sapers, 2000). Its use may be limited to produce that contains endogenous catalase activity to remove residual HP, as mandated by the FDA. Sapers et al. (1999) investigated the use of HP to reduce populations of *E. coli* on the surfaces of apples. The treatment was effective in removing or killing *E. coli* cells located on the surface (3 to 4 \log_{10} reduction), but remained largely ineffective in reducing numbers associated with the stem and calyx end of apples (1 to 2 \log_{10} reduction), indicating that microorganisms located in cores of apples were afforded protection from the action of the sanitizer.

Peroxyacids

Use of PAA for sanitizing raw produce has shown promise as an effective alternative to chlorine-based sanitizers. In their comparison of several sanitizers to kill *E. coli* O157:H7 cells associated with apples surfaces, Wright et al. (2000) determined that 80 ppm PAA outperformed 200 ppm hypochlorite, 5% acetic acid followed by 3% hydrogen peroxide, and a phosphoric acid fruit wash. In this study, PAA reduced *E. coli* O157:H7 by 3.1 \log_{10} . In a separate study, 80 ppm PAA was determined to reduce populations of *Salmonella* and *E. coli* O157:H7 on cantaloupes, honeydew melons, and asparagus by a range of 1.4 to 3.3 \log_{10} and 0.79 to 2.9 \log_{10} , respectively.

The antimicrobial efficacy of a mixture of PAA and peroxyoctanoic acid for the microbiological control of fruit and vegetable process water has been evaluated (Hilgren and Salverda, 2000). Results suggested that the combined peroxyacid treatment was effective in controlling the growth of microorganisms in water and performed better than the PAA alone. It was concluded that the use of PAA may be preferred over other commonly used antimicrobial agents such as hypochlorite because this agent has environmentally friendly decomposition by-products (i.e., oxygen, acetic acid, octanoic acid, and water) and exhibits greater stability in the presence of organic material.

Ozone

Use of ozone to improve the safety and extend the shelf life of raw fruits and vegetables has been reviewed (Kim et al., 1999; Xu, 1999). Treatment with gaseous ozone at low concentrations extended the shelf life of some fruits and vegetables, especially those that are easily injured by water. The increase in the shelf life of some fruits may be attributed to the oxidation of ethylene and to the

removal of other metabolic products of ozone (Kim et al., 1999). Inactivation of microorganisms associated with fruits and vegetables certainly, however, contributes to this observation.

Sanitization treatments of produce with ozone demonstrate its potential as an alternative to chlorine (Kim et al., 1999; Xu, 1999). Achen and Yousef (2001) observed reductions in populations of *E. coli* O157:H7 on apple surfaces following treatment with aqueous ozone of 3.7 log₁₀. They concluded that treatments were more effective when ozone was bubbled during apple washing than by dipping in preozonated water. Moreover, decontamination of apples was optimal when fruits were pretreated with a wetting agent prior to bubbling ozone for 3 minutes in the wash water.

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17 Indirect and Miscellaneous Antimicrobials

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CONTENTS

Sodium Chloride	574
Chemistry and Production	574
Antimicrobial Activity	575
Mechanism of Action.....	577
Applications	577
Toxicology.....	578
Assay	578
Polyphosphates.....	578
Chemistry	578
Antimicrobial Activity	580
Mechanism of Action.....	580
Applications	581
<i>C. botulinum</i>	581
Other Clostridia.....	581
<i>S. aureus</i>	582
Other Foodborne Pathogens.....	582
Spoilage Bacteria	582
Mold.....	582
Treatments with Combinations Containing Phosphates.....	583
Regulatory Status	583
Assay	583
Ethylenediaminetetraacetic Acid.....	583
Physical and Chemical Properties	584
Antimicrobial Activity	584
Mechanism of Action.....	585
Applications	586
Toxicology.....	587
Regulatory Status	587
Assay	587
Sodium Bicarbonate.....	588
Chemistry	588
Antimicrobial Activity	588
Applications	588
Mechanism of Action.....	589
Regulatory Status	589
Assay	589

Ethanol	589
Physical and Chemical Properties	589
Antimicrobial Activity and Mechanism of Action	589
Applications	590
Toxicology	590
Regulatory Status	590
Assay	590
Propylene Glycol	591
Chemistry and Production	591
Antimicrobial Activity and Mechanism of Action	591
Toxicology	591
Regulatory Status	591
Diacetyl	592
Antimicrobial Activity	592
Mechanism of Action	592
References	592

Indirect food antimicrobials are compounds that are added to foods for purposes other than their antimicrobial effects. Some of these food compounds may not produce antimicrobial effects by themselves, but they potentiate or intensify the inhibitory effects of other preservatives or preservative systems. The possibility that certain of these food components antagonize the activity of an antimicrobial cannot be ignored. As a result, evaluation of potential antimicrobial effects requires that in addition to testing the individual ingredient, interaction in the complete food system must be examined. Many indirect antimicrobials, which look promising in tests conducted in culture media, partially or totally lose their effect in complex food systems, whereas proteins, micronutrients, and other factors serve to protect the microorganisms.

Interest in indirect antimicrobial compounds is enhanced by the fact that many of these components are classified as generally recognized as safe (GRAS) and approved by regulatory agencies. The effects of their addition on food attributes have already been established. GRAS indirect antimicrobials are primarily used as antioxidants, sequestrants, buffering and defoaming agents, emulsifiers, flavor substances, and adjuvants. Those discussed in this chapter include sodium chloride, polyphosphates, EDTA, sodium bicarbonate, ethanol, propylene glycol, and diacetyl. Several others that fall into the category of indirect antimicrobials are treated in other chapters.

SODIUM CHLORIDE

Sodium chloride, also called salt, table salt, and rock salt, has been used as a food additive for thousands of years. During the Middle Ages this important food preservative and flavorant became a trading commodity. For centuries salt remained the most important preservative used for meat, fish, and vegetables (Leuck, 1980). Salt continues to be used as a preservative, but it is most commonly used in combination with other antimicrobials or preservation techniques.

CHEMISTRY AND PRODUCTION

NaCl (molecular weight 58.44) is readily soluble in water at neutral pH values. A total of 1 g dissolves in 2.8 ml water at 25°C. A saturated solution of common salt contains 26.5 g NaCl per 100 g water (31.1 g per 100 ml) and has a density of 1.202. The salt has a melting point of 80°C, boiling point of 1413°C, and a density of 2.165 (Lewis, 1989). Primarily sodium chloride, common salt may also contain some calcium and magnesium chloride.

TABLE 17.1
Water Activity of Common Salt Solutions

a_w value	Content of Solution (g NaCl per 100 g H ₂ O)
0.995	0.88
0.99	1.75
0.98	3.57
0.96	7.01
0.95	8.82
0.94	10.34
0.92	13.50
0.90	16.54
0.88	19.40
0.86	22.21
0.85	23.55
0.84	24.19
0.82	27.29
0.80	30.10
0.78	32.55
0.76	35.06
0.75	36.06

Source: From Robinson and Stokes (1959).

Salt production involves either mining or evaporation. Mined salt is essentially sterile, except for microorganisms added through mining procedures (Ayers et al., 1980). Salt produced through evaporation of sea water is called solar salt and may be contaminated with halophilic and haloduric microorganisms (Ayers et al., 1980).

ANTIMICROBIAL ACTIVITY

Salt lowers the water activity a_w of solutions, which is likely the primary cause for inhibiting microbial growth. As seen in Table 17.1, to achieve a_w of 0.90, which inhibits most bacteria, a salt solution of 16.5% in the water phase is required. This salt concentration is unacceptable in many applications because of taste restrictions. Thus, NaCl is most often used in combination with other additives and preservation techniques. The relationship between a_w and salt concentrations in water is shown in Table 17.1 (Lueck, 1980).

NaCl at concentrations of 0.85% to 0.90% in water produced isotonic conditions for nonmarine microorganisms (Jay, 2000). Ayers et al. (1980) reported classification of bacteria into several categories of salt tolerance. The “nonhalophiles” grow best in the presence of 0% to 0.5% NaCl but may tolerate much higher levels. Nonhalophiles include some members of clostridia, enterococci, and staphylococci groups. The “slight” halophiles grow in the presence of 1.5% to 5.0% NaCl and include some species of *Pseudomonas*, *Vibrio*, *Achromobacter*, and *Flavobacterium*. “Moderate” halophiles tolerate NaCl solutions of 5% to 20% and include lactic acid bacteria and some spore formers. Obligate halophilic bacteria require NaCl concentrations of 15% and grow well in brines that approach saturation. Organisms that can survive in higher concentrations of salt but do not grow in these concentrations are referred to as haloduric. Most nonmarine bacteria are inhibited by 20% NaCl or less; molds generally tolerate higher levels (Jay, 2000).

Food poisoning bacteria have been the focus of most of the research on salt tolerance. Staphylococci grow at lower a_w than most other nonhalophilic bacteria. Growth has been demonstrated at a_w as low as 0.83 under ideal conditions, but the generally recognized minimum a_w is 0.86 (Jay,

2000). Increasing the salt level has the effect of raising the minimum pH for growth. Temperature is also an important parameter for determining growth at various salt concentrations. Enterotoxin production by *Staphylococcus aureus* may be more susceptible to NaCl than growth. As little as 4% salt reduced staphylococcal enterotoxin B (SEB) production in *S. aureus* by 80%, and 10% salt totally inhibited production (McLean et al., 1968). Troller and Stinson (1978) showed that growth of *S. aureus* 196E and production of SEB were reduced when the NaCl concentration was increased from 0% to 5.3%, but production of staphylococcal enterotoxin A (SEA) was unchanged. Pereira et al. (1982) and Smith et al. (1987) showed a reduction in SEA production as salt concentrations were increased from 0% to 4% and from 0% to 10%, respectively. Robach and Stateler (1980) showed that NaCl at 7% had only a slight effect on the growth of two strains of *S. aureus*, but a combination of 7% NaCl and 0.2% potassium sorbate significantly inhibited growth at pH 6.0. However, LaRocco and Martin (1987) did not observe this synergistic effect after 15 days at 22°C or 48 hours at 35°C using combinations of 2%, 3%, and 7% NaCl with 0.2% and 0.3% potassium sorbate at pH 6.3. Stern et al. (1979) found that 5% and 7% NaCl combined with 50 ppm butylated hydroxyanisole (BHA) resulted in an extended lag phase or total inhibition of *S. aureus* at pH 6.0 and 7.0.

The anaerobic spore formers *Clostridium perfringens* and *Clostridium botulinum* are significantly inhibited by salt. The limiting salt concentration for the growth of most strains of *C. perfringens* has been reported to be 7% to 8% (Craven, 1980). The lowest a_w for growth with salt used as a solute was reported to be 0.95 to 0.97. Robach (1980) found that 5% NaCl in combination with 0.1% potassium sorbate caused total growth inhibition of *C. sporogenes* PA 3679 at 24°C and 37°C. *C. botulinum* spores and vegetative cells of types A and B have been shown to be inhibited by 10% NaCl, whereas type E growth was inhibited at 5.5% NaCl (Sofos et al., 1979). A water-phase NaCl concentration of 3.5% to 3.8% was required to inhibit type E toxin production in hot-process whitefish and salmon steaks held at 25°C, depending on the inoculum size (Pelroy et al., 1982, 1985). In combinations of NaCl, sodium acid pyrophosphate, and potassium sorbate, growth from spores of *C. botulinum* strain 52A was more inhibited with treatments containing 0% NaCl than with levels of 1.25% and 2.5%. Because salt concentrations in most cured meat products are lower than the minimum inhibitory levels just discussed, salt by itself is not a practical inhibitor of *C. botulinum*. Interactions of salt with pH, nitrite, heat, type of meat, and spore level in cured meats are discussed in Chapter 6.

Other foodborne pathogens have also been investigated for their NaCl resistance. The minimum a_w for salmonellae has been reported as below 0.94 in media with neutral pH. Minimum a_w values are higher as the pH is decreased. This interaction of parameters was demonstrated by a study of the effects of NaCl concentration, pH, and temperature in ground pork (Alford and Palumbo, 1969). Higher concentrations of NaCl were required to inhibit growth as temperature and pH increased: 8.0% NaCl at pH 6.5 and 20°C or 30°C versus 3.5% NaCl at pH 5.0 and 10°C. *Bacillus cereus* is able to grow at 7.5% but not 10% NaCl and is limited by an a_w of 0.95. *Vibrio parahaemolyticus* is moderately halophilic, requiring at least 0.25% NaCl, with an optimum of 2% to 3% NaCl (Beuchat, 1982); the organism is rarely able to grow at 10% NaCl. Decreased growth rate was observed for *Shigella flexneri* at 0.5% NaCl in brain heart infusion (BHI) broth at pH 5.5 and 28°C (Zaika et al., 1989). *Campylobacter jejuni* has been found to grow at 0%, 0.5%, and 1% NaCl in brucella broth at 42°C; however, cell counts declined with 2% and 3% NaCl (Doyle and Roman, 1982; Abram and Potter, 1984). NaCl concentrations of 4.5% and higher extended the generation time of *Escherichia coli* O157:H7 in tryptic soy broth (TSB), and 8.5% was inhibitory (Glass et al., 1992). In a more recent study, 3.5% NaCl in BHI broth was not inhibitory to a bioluminescent strain of the pathogen (Tomicka et al., 1997).

Among foodborne pathogens, *Listeria monocytogenes* is second only to the staphylococci in its ability to grow at a_w values <0.93, and the minimum a_w that permitted growth with salt was 0.92 (Farber et al., 1992). Growth of two strains of *L. monocytogenes*, Scott A (serotype 4b) and Brie 1 (serotype 1b), was investigated at 4°C and 30°C, using NaCl, glycerol, and sucrose as test solutes.

The strains did not grow in NaCl-supplemented TSB at a_w 0.90, and the tolerance to low a_w was markedly lower at 4°C than at 30°C (Tapia de Daza et al., 1991).

As with bacteria, yeasts and molds vary greatly in their tolerance to sodium chloride. For example, several genera of yeasts, including *Debaryomyces*, *Candida*, *Rhodotorula*, *Hansenula*, and *Pichia*, are responsible for the softening of pickles in brines of up to 20% salt. Beuchat (1981a, b) examined the effect of water activity reducing solutes in combination with heat on the death of yeasts and molds. Of six yeast strains examined (*Debaryomyces hansenii*, *Pichia membranaefaciens*, *Saccharomyces cerevisiae*, *Candida krusei*, *Kloeckera apiculata*, and *Rhodotorula rubra*), all except *R. rubra* showed increased resistance to heat (46°C to 54°C) in the presence of 3% salt. In the presence of 12% NaCl, only *D. hansenii* had enhanced heat stability over the control. Beuchat (1981a) also tested NaCl (0%, 3%, 6%, 9%, and 12%) plus heat in a similar manner with four mold species: *Aspergillus flavus* (conidia), *Penicillium puberulum* (conidia), *Geotrichum candidum* (vegetative cells), and *Byssoschlamys nivea* (ascospores). All species except *P. puberulum* had increased resistance to heat at 3% NaCl. At 12% NaCl only *B. nivea* ascospores showed heat resistance greater than the control. In contrast to the findings of Beuchat (1981a), Doyle and Marth (1975) found that NaCl at concentrations of up to 16% increased the heat resistance of *A. flavus* and *Aspergillus parasiticus* conidia. Relatively high concentrations of 6%, 8%, or 10% NaCl in glucose-yeast-salt medium produced slower mold growth and low aflatoxin production in *A. parasiticus* (El-Gazzar et al., 1986).

MECHANISM OF ACTION

Several reasons have been suggested for the inhibition of microorganisms by salt. The primary reason is probably cellular plasmolysis. Smith et al. (1987) found that NaCl inhibited respiration, o-nitrophenyl- β -galactoside hydrolysis, and glucose utilization, suggesting that it prevents transport of substrate into the cells of *S. aureus* 196E. They suggested that the cells waste energy by excluding sodium ions, and thus less energy is available for transport across the cell membrane. Other possible mechanisms include limiting oxygen solubility and interference with enzymes.

As protection against osmotic stress, microorganisms accumulate solutes such as proline, glutamate, glutamine, γ -amino butyrate, alanine, glycine betaine, sucrose, trehalose, glucosylglycerol, and K^+ ions. The role of proline as an osmoprotectant was demonstrated by Christian (1955a, b), who showed that addition of the amino acid relieved bacterial growth inhibition in media with elevated osmolarity. A proline-overproducing mutant of *Salmonella enterica* serovar Typhimurium exhibited increased salt tolerance (Csonka, 1981), and the role of proline as an effective osmolyte has been demonstrated in several bacteria, including *Listeria* (Bayles and Wilkinson, 2000, Beumer et al., 1994, Sleator et al., 2001).

Patchett et al. (1992) studied the effect of NaCl on the intracellular pools of *L. monocytogenes* and observed accumulation of osmoprotectants (K^+ ions, betaine, and glutamate), but not of proline, when the organism grew on culture media. The cellular concentration of amino acids, particularly of glycine and alanine, increased when NaCl concentration in the media increased from zero to 7.5% (Patchett et al., 1992).

APPLICATIONS

Salt is generally included in food formulations for flavor rather than as a preservative. In the presence of other preservatives, however, salt may exhibit synergistic effects. Foods in which salt plays a role in preservation include cheese, fish, cured meats, liquid eggs, and fermented vegetables. For example, butter and margarine contain approximately 2% salt by weight. Because these products contain only about 16% water, the concentration of the salt in the water phase can be as high as 12.5%. This amount of salt would inhibit most microorganisms, especially at refrigerator temperatures.

Because dietary sodium has been implicated as having a role in the development of hypertension in some individuals, public health authorities have recommended reducing the intake of sodium chloride (Expert Panel, 1980). This warning has prompted processed meat manufacturers to investigate reduced salt preservative systems. The combination of 0.26% potassium sorbate with 0.65% and 1.30% NaCl significantly delayed growth of *C. perfringens* at 20°C in extruded processed chicken and turkey products, but these low levels of salt alone were not effective (Sofos, 1986). Madril and Sofos (1986) found that currently used NaCl concentrations of 4.1% are important in delaying microbial growth in comminuted beef-pork products stored at 27°C, especially at pH values of 6.0 or lower. When NaCl was reduced to 2.3% brine, shelf life was significantly shortened even at pH 5.7. Sodium acid pyrophosphate, added at 0.5% in addition to the sorbate, improved shelf life in the low-salt product. The shelf life of vacuum-packaged beef steaks was extended using an aqueous dip consisting of 10% potassium sorbate, 5% NaCl, 10% sodium acetate, and 5% of a commercial mixture of phosphates (Unda et al., 1990).

TOXICOLOGY

The oral median lethal dose (LD₅₀) of common salt in rats was determined to be 3.75 g/kg body weight (Lueck, 1980). Lueck also reported that the oral LD₅₀ of concentrated aqueous solutions of NaCl was 2.7 g/kg in fasted rats and 6.1 g/kg for nonfasted rats over a 100-day period. In humans, sodium is essential for the maintenance of osmotic pressure of body fluids, permeability of cellular membranes, and control of acid–base equilibrium. The safe and adequate dietary intake level of sodium for adults is 1.1 to 3.3 g/day, which is equivalent to 2.8 to 8.4 g salt per day (Darby, 1980). Salt is excreted through the kidneys and skin. Daily renal losses of 6.0 to 12.5 g are normal, whereas sweat losses are variable (Darby, 1980).

ASSAY

NaCl can be assayed in foods using volumetric or potentiometric titration as described in Official Methods of Analysis (AOAC, 1984, 1990).

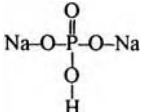
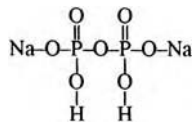
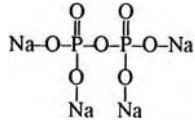
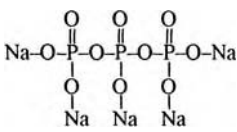
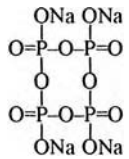
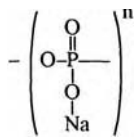
POLYPHOSPHATES

Polyphosphates (polyP) are negatively charged strong polyanions that form complexes with metal ions and with positively charged macromolecules, such as proteins. A large number of these compounds, ranging from the pyrophosphates to the hexametaphosphates, with chain lengths of more than 10 PO₄ groups, have numerous applications in foods. These compounds and their structures are shown in Table 17.2. The major functions of polyphosphates in foods are summarized in Table 17.3. Some of these functions are related to the ability of the compounds to form soluble complexes with metal ions, which is beneficial in preventing or retarding undesirable effects, such as lipid oxidation, and in providing mineral supplementation (e.g., iron and calcium in cereal products) (Ellinger, 1972). Meat products often contain polyphosphates, which serve to stabilize meat emulsions, increase tenderness and hydration, retain color, and improve flavor. Dairy products, such as processed cheese, custard, and pudding, also contain phosphates. Their major function is to promote the dispersion of food constituents and stability. Polyphosphates also function in acidification and water softening (Molins, 1991).

CHEMISTRY

Phosphates are prepared from phosphoric acid neutralized with alkali metal ions, such as sodium, potassium, or calcium. The orthophosphates (PO₄³⁻), frequently used in foods, lack antimicrobial activities. The condensed phosphates, produced by dehydration at elevated temperatures of mixtures of orthophosphates, include the straight-chain (polyphosphates) and the ring structure compounds

TABLE 17.2
Phosphate Compounds Used in Foods

Compound	Formula	MW	Structure
Disodium phosphate (DSP)	Na_2HPO_4	142	
Sodium acid pyrophosphate (SAPP)	$\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$	222	
Tetrasodium pyrophosphate (TSPP)	$\text{Na}_4\text{P}_2\text{O}_7$	266	
Sodium tripolyphosphate (STPP)	$\text{Na}_5\text{O}_5\text{O}_{10}$	368	
Sodium tetrametaphosphate (STMP)	$m(\text{NaPO}_3)_4$	408	
Sodium hexametaphosphate (SHMP) ($n = 13, 15, 21$)	$(\text{NaPO}_3)_n$	1326 ($n = 13$) 1530 ($n = 15$) 2142 ($n = 21$)	

(metaphosphates). The straight-chain compounds consist of the pyrophosphates, with two phosphorus atoms; tripolyphosphates, with three P atoms; and the long-chain polyphosphates, with four or more P atoms. The first two are crystalline; the other polyphosphates are amorphous or glassy. The long-chain polyphosphates are mixtures of varying chain-length phosphates (Ellinger, 1972; Van Wazer, 1971).

Polyphosphates are characterized by a highly charged anionic nature. Because of their geometry, a chelate ring can form between the oxygen atoms from adjacent PO_4 groups and a polyvalent metal ion. Chain phosphates form far more stable complexes with polyvalent cations than the ring structure phosphates, being hindered sterically from assuming all possible configurations (Van Wazer and Callis, 1958).

TABLE 17.3
Major Functions of Phosphates in Foods

Cereal products:	Promote iron and calcium supplementation
Meat products:	Stabilize meat emulsions
	Increase tenderness
	Increase hydration and yield
	Retain color
	Improve flavor
Dairy products:	Promote dispersion of proteins
	Buffering

ANTIMICROBIAL ACTIVITY

Suppression of microbial growth by polyphosphates has been reported in recent years by a number of investigators (Ellinger, 1972; Firstenberg-Eden et al., 1981; Molins et al., 1984; Nelson et al., 1983; Shelef, 1986; Tompkin, 1984; Wagner and Busta, 1985; Jen and Shelef, 1986). The effect on microbial toxins has also been studied, particularly in relation to *C. botulinum* toxins (Wagner, 1986) but also in relation to *S. aureus* (Thomas and Wagner, 1987; Shelef and Wang, 1989; Shelef et al., 1990).

In regard to *C. botulinum*, Seward et al. (1982) showed that the addition of 0.5% tripolyphosphate to media containing 1.5% sorbate at pH 7.1 prevented normal cell growth. Wagner and Busta (1983) reported delayed *C. botulinum* toxicity in peptone-yeast extract-glucose broth containing 0.4% sodium acid pyrophosphate. Other investigators reported effects of polyphosphates alone and in combination with antimicrobials, such as sorbate and nitrate, on *C. botulinum* in culture media. These studies were reviewed by Tompkin (1984) and Wagner (1986). In evaluating the published reports, Gram-positive bacteria appear to be generally more susceptible to polyphosphates than are Gram-negative bacteria. Growth inhibition of *Pseudomonas* species in broth required in excess of 0.5% sodium tripolyphosphate (STPP) (Zessin and Shelef, 1988), and the organisms were not inhibited in foods, such as ground beef or pork, by less than 1%.

MECHANISM OF ACTION

The antimicrobial activity of polyphosphates is generally believed to result from their ability to chelate cations essential for growth. Two observations support the association with cation chelation. Gram-positive bacteria have been consistently more sensitive to polyphosphates than Gram-negative organisms. These bacteria have higher cation requirements compared to Gram-negative bacteria and Ca^{2+} and Mg^{2+} are essential for the structural integrity of their cell wall. In addition, higher concentrations of the compounds have been reported for effects in foods, in which polyvalent cation levels are generally higher than in culture media (Shelef et al., 1990; Zaika et al., 1997). For example, comparison of concentrations of four polyvalent cations in BHI broth and minced beef showed that they are between 13 and 57 times higher in the latter (Shelef, 1988). As a result, a high concentration of polyphosphates is required to produce cation-deficient conditions in meat. In an examination of the minimum inhibitory concentration of sodium tripolyphosphate in three liquid media (nutrient broth, BHI, and TSB), the sensitivity of bacteria was shown to decrease with an increase in the mineral content in the media (Zessin and Shelef, 1988). Confirmation that inhibition was caused by chelation of essential cations was provided when inhibition was reversed by media supplementation with divalent cations, particularly magnesium. This has been shown in studies with yeast, pseudomonads, *S. aureus*, and *Bacillus cereus* (Vishniac, 1950; Elliott et al.,

1964; Jen and Shelef, 1986; Lee et al., 1994b; Maier et al., 1999). Moreover, the orthophosphates, which do not chelate cations, did not inhibit mold, Gram-positive, or Gram-negative organisms (Knabel et al., 1991).

Wagner and Busta (1985) noted morphologic changes in vegetative cells of *C. botulinum* grown in SAPP and delayed toxicity in peptone-yeast extract-glucose broth containing 0.4% SAPP. The authors suggested that the production or function of protease responsible for toxin activation might have been inhibited in the presence of the compound. Lee and coworkers (1994a, 1994c) concluded that the lytic effect on *S. aureus* by polyphosphates results from binding of the compounds to the cell wall and chelation of structurally essential metals, which then destabilizes the cell walls leading to lysis. Matsuoka et al. (1995, 1997) observed only bacteriostatic effect on *S. aureus* by sodium hexametaphosphate (SHMP) and suggested that the polyP caused leakage of magnesium from the cells, loss of osmoregulation, and membrane damage. Using *B. cereus* as the test organism, Maier et al. (1999) observed bactericidal effects by 0.1% polyP or higher on log-phase cells but not on stationary-phase cells. Germination and outgrowth of spores were also inhibited, and sporicidal effects were observed at concentrations of 1%. Mg^{2+} and Ca^{2+} reversed the antimicrobial activity. Elongated cells were formed in the presence of sublethal polyP concentrations. The authors concluded that polyP may have an effect on FtsZ, the bacterial cell division protein, whose GTPase activity depends on divalent metals. Cell elongation and changes in cellular morphology were also reported in *L. monocytogenes* and other organisms (Zaika et al. 1997).

Polyphosphate is found in bacterial, fungal, plant, and animal cells and has been shown to have numerous biological functions, including substituting for adenosine triphosphate in kinase reactions, providing a reservoir for P_i , chelating metals, and other functions. It has a regulatory role in bacterial adaptations for stationary-phase survival and is essential for the motility and virulence of some bacterial pathogens. PolyP kinase (PPK) is specific for synthesizing and metabolizing polyphosphate, and there are indications that the enzyme is needed for virulence of *Salmonella enterica* species and other pathogens (Kornberg et al., 1999).

APPLICATIONS

C. botulinum

Addition of 0.4% SAPP to beef-pork frankfurter emulsions containing nitrite (40 ppm) and sorbate (0.26%) delayed botulinal toxin production, and the number of toxic samples was less than in formulations without the phosphate (Wagner and Busta, 1983). Additional studies on the effect of polyphosphates in combination with the antimicrobials nitrate and sorbate on *C. botulinum* have been reported, and the antibotulinal effects of phosphates in cured meats have been reviewed (Tompkin, 1984; Wagner, 1986). Polyphosphates delayed botulinal toxin production in pasteurized process cheese spread with 60% moisture and inhibited it in spreads with lower moisture content (Eckner et al., 1994).

Other Clostridia

The survival and growth of inoculated *C. sporogenes* PA 3679 and of natural aerobic and anaerobic bacterial flora were studied in cooked vacuum-packed bratwurst containing 0.5% polyphosphates (Molins et al., 1985a). Of the four compounds tested, none had any effect during refrigerated storage, but SAPP inhibited aerobic and anaerobic bacteria, including *C. sporogenes*, on temperature abuse at 25°C for 48 hours. Madril and Sofos (1986) demonstrated that SAPP improved the shelf life of low-salt comminuted meat products that had been inoculated with spores of *C. sporogenes* more than expected from pH effects alone. *C. tyrobutyricum* in processed cheese spreads was controlled by 0.5% polyphosphate, and 1% totally inhibited the organism (Loessner et al., 1997).

S. aureus

The effect of four compounds (SAPP, STPP, TSPP [tetra sodium pyrophosphate], and SHMP) on aerobic, mesophilic, and psychrophilic bacterial growth and on the survival of *S. aureus* was investigated in refrigerated uncooked bratwurst (Molins et al., 1985b). No significant inhibition was found, although addition of SAPP resulted in consistently lower total aerobic plate counts. The effects of polyphosphates on *S. aureus* growth and enterotoxin A production using enzyme-linked immunosorbent assay were studied in custard and beef (Shelef et al., 1990). Reduced cell numbers were seen only in custard with SAPP at pH 5.5.

Other Foodborne Pathogens

A process using trisodium phosphate (TSP) to reduce viable *Salmonella* species on chicken carcasses has been approved by the U.S. Department of Agriculture (USDA) (Giese, 1993). The process was effective in reducing *E. coli* (Giese, 1993), *Campylobacter* (Slavik et al., 1994), *Salmonella* (Kim et al., 1994), and *S. aureus* (Lee et al., 1994a). Of 10% solutions of STPP, monosodium phosphate (MSP), SAPP or SHMP, and 1% TSP, the latter was most effective in reducing viable populations of *Salmonella* species, *L. monocytogenes*, and psychrotrophs on chicken skin after washing for 30 minutes (Hwang and Beuchat, 1995). Effects of rinses containing phosphates on meat and poultry carcasses inoculated with *S. Typhimurium* and *E. coli* O157:H7 were also reported, and reductions of the pathogens depended on the type of compounds and treatments (Kim and Slavik, 1994, Rathgeber and Waldroup, 1995). Treatments of fresh ground beef or pork with phosphates were generally ineffective, evidently because of degradation of the compounds by the meat phosphatases (Flores et al., 1996).

SPOILAGE BACTERIA

Elliott et al. (1964) reported growth inhibition of nonfluorescent pseudomonads and extended shelf life after chilling poultry overnight on ice containing 3% to 8% polyphosphates. Fluorescent strains were not affected. Addition of magnesium ions reversed the inhibition. Spoilage microflora in fresh beef was not affected by polyphosphate in concentrations of 0.5%, although the shelf life of fresh ground pork was extended with 1% pyrophosphate (Molins et al., 1987). Nielsen and Zeuthen (1983) reported studies using selected spoilage bacteria in vacuum-packed sliced bologna-type sausage during refrigerated storage. The tested compounds included a mixture of SAPP, STPP, and SHMP with an acid pH or STPP. *Brochothrix thermosphacta* and *Serratia liquefaciens* were the test organisms, and these bacteria were strongly inhibited by the low-pH phosphate mixture during storage under vacuum at 2°C and 8°C. Marshall and Jindal (1997) tested the effects of TSP, STPP, and sodium metaphosphate (SMP) dipping solutions on the microbiological quality of catfish frames after removal of the fillets. TSP was most effective in reducing surface microbial numbers.

MOLD

Post et al. (1968) reported the effect of phosphates on fungi and preservative effects on fresh cherry fruit. Mold growth and mycotoxin production studied in high-moisture (20%) corn treated with a number of different polyphosphates showed that acid sodium hexametaphosphate, TSPP, and STPP totally prevented or reduced mold growth when added in powder form at 1% or 2% (wt/wt). Application of 2% acid SHMP and TSPP in spray form was also effective. Treatment of moldy corn with SHMP or TSPP inhibited aflatoxin production (Lebron et al., 1989). In a study with four phosphate compounds (1% to 2%), production of aflatoxin B₁ and G₁ was reduced in Sabouraud dextrose agar but not in frankfurters (Marsh et al., 1996).

TREATMENTS WITH COMBINATIONS CONTAINING PHOSPHATES

There are numerous reports on preservative effects in foods by combinations of chemicals and treatments that include polyphosphates. The majority of the foods consist of meat, poultry, and fish. For example, dipping the Australian fish morwong in a solution of 1.2% potassium sorbate and 10% polyphosphate, followed by packaging in 100% CO₂, produced an extended shelf life (Statham et al., 1985). Reduction of chicken skin-attached microorganisms, including *S. aureus* and other foodborne bacterial species, was observed after treatment with TSP, followed by exposure to lysozyme and nisin (Carneiro de Melo et al., 1998). Although the inclusion of polyphosphates in such mixtures of chemicals is generally not designed for antimicrobial purposes, sufficient evidence has accumulated to confirm that these compounds also contribute to the increased microbial stability of the treated foods.

Several factors may affect the degree of microbial inhibition by polyphosphates in foods. First, the presence of polyphosphatases and pyrophosphatases in fresh muscle cells may cause rapid hydrolysis of the compounds to the inactive ortho form. This may explain reports on the lack of inhibitory effects of condensed phosphates in fresh meats, particularly in minced meats, in which the rate of hydrolysis is more pronounced. Antimicrobial effects were observed in heat-treated meats, evidently because heat processing inactivates these enzymes. Second, the effect of polyphosphates on the pH of a food system plays an important role in growth inhibition. The low-pH pyrophosphates appear to be superior to other condensed phosphates in foods, and a pH-lowering effect of 0.3 units or more significantly enhanced the antimicrobial effectiveness, although it was not totally responsible for it. Finally, the polyvalent cation content is a factor that may affect the degree of inhibition; inhibition is significantly reduced or completely disappears in high-cation foods.

REGULATORY STATUS

The polyphosphates are general-purpose and multiple-purpose GRAS substances (21 CFR 182.1087, 582.1087, and others) (CFR, 1990). Additions to meat and poultry products are regulated by the USDA, and levels of 0.5% by weight are generally allowed in the finished products. Phosphates are not allowed in fresh meat and poultry products.

ASSAY

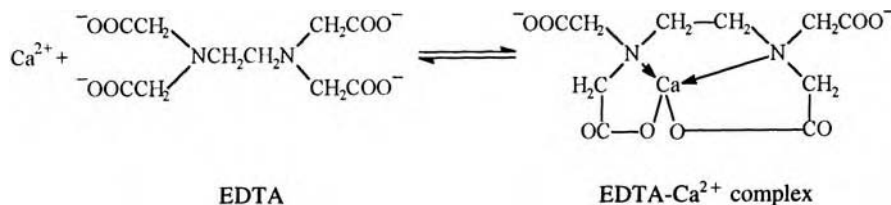
Upon hydrolysis, pyrophosphates and polyphosphates form orthophosphate, which can be determined by a number of methods used for total phosphorus analysis. A colorimetric method is based on the reaction of orthophosphate and molybdate ions, which produces a blue color (Murphy and Riley, 1962). This method was further modified by Dick and Tabatabai (1977). Assays for the specific compounds are described in the Food Chemicals Codex (National Academy of Sciences, 1981).

ETHYLENEDIAMINETETRAACETIC ACID

EDTA (versene) belongs to a class of compounds known as chelating, sequestering, or metal-complexing agents. The most thoroughly studied group of chelating agents is the family of polyaminocarboxylic acids, EDTA being its most widely used member. EDTA reacts with alkaline earth and heavy metals to form metal complexes, thereby removing reactive multivalent cations from solution (Klaassen, 1980). In foods, EDTA is added primarily to eliminate by chelation trace metals that act as prooxidants. The destruction and inhibition of bacterial cells by chelating agents are well recognized.

PHYSICAL AND CHEMICAL PROPERTIES

EDTA reacts with metals to form a complex. For chelation, the ligand or sequestrant must have the proper steric and electronic configuration and be at the optimal ionic strength and pH. A high-stability chelate forms with calcium:



EDTA becomes increasingly dissociated as the pH rises and the quantity of complexed metal increases. A wide range of metal ions are sequestered, including those of Al, Ni, Fe, Cu, Co, Zn, Ba, Ca, Fe, Mg, Mn, and Sr.

The use of EDTA in foods has been limited to the disodium salt ($\text{Na}_2\text{H}_2\text{EDTA}$) and the disodium-calcium complex (Na_2CaEDTA) at levels of 50 to 150 ppm (Furia, 1964). The salts are available as spray-dried powders, which in use concentrations are colorless and freely soluble in water, with only a faint saline taste. Chelation of iron and copper is particularly desirable in a variety of foods because traces of these metals act as prooxidants in fat-containing foods that are prone to oxidation and also cause discoloration in vegetables, fruits, and other foods.

ANTIMICROBIAL ACTIVITY

EDTA may act as a direct inhibitor of several species of organisms or may act synergistically with other antimicrobial agents to promote bacterial destruction. There is extensive literature, which dates back to the 1960s, on inhibition of Gram-negative bacteria by EDTA, particularly of *Pseudomonas aeruginosa*. MacGregor and Elliker (1958) reported that 250 ppm EDTA destroyed 99.999% of a population of *P. aeruginosa*. Gray and Wilkinson (1965) found that EDTA at an alkaline pH selectively solubilized a high proportion of the cell-wall carbohydrate and phosphorus in a number of Gram-negative bacteria and was bactericidal for *P. aeruginosa* in 0.001 M solution, destroying more than 99.99% of the cells in suspension.

Gram-negative organisms, normally resistant to the action of lysozyme, were found to be lysed in the presence of EDTA. In a study by Repaske (1958), lysis of *Azotobacter vinelandii*, *Escherichia coli* B, *Pseudomonas fluorescens*, and *P. aeruginosa* was rapid in the presence of EDTA, lysozyme, and Tris buffer. The maximum extent and rate of lysis were shown to vary with the pH of the lysing medium. Shively and Hartsell (1964a) found EDTA in distilled water to be an active lytic agent against 10 species of *Pseudomonas*. With the addition of lysozyme, lysis was increased both additively and synergistically. Various chemical treatments altered the lytic response of the pseudomonads to an EDTA-lysozyme-buffer system. Under acid conditions (pH 6.0) lysis was impaired; alkaline conditions (pH 8.0) restored lytic action. Lysis was inhibited by NaCl (0.1% to 2.0%) and 10% starch or sucrose (Shively and Hartsell, 1964b).

Robach and Stateler (1980) found the addition of EDTA to act synergistically with potassium sorbate against *S. aureus*. Used in combination, 0.2% sorbate and 50 ppm EDTA proved to be an effective inhibitory system.

Some bacterial species, including *E. coli* and *Serratia marcescens*, have the ability to develop resistance to the action of quaternary ammonium compounds (QAC), common sanitizing agents. MacGregor and Elliker (1958) found that QAC-resistant cells of *P. aeruginosa* could be made

sensitive by treatment with EDTA. EDTA was also found to enhance glucose inhibition of *C. perfringens* in a sugar and amino acid medium (Schroder and Busta, 1974).

An investigation into the effect of EDTA on heat resistance of *C. sporogenes* PA3679 spores in phosphate buffer (pH 7.0) and in white asparagus puree (pH 4.5 to 5.8) showed that EDTA was most active on spores subjected to more severe heat treatments and at the higher pH levels (Silla Santos and Torres Zarzo, 1997). Phillips and Duggan (2001) studied the effect of EDTA and TSP, alone and in combination with nisin, on growth of *Arcobacter butzleri*, a common contaminant of foods of animal origin. EDTA alone (1 to 20 mM in culture media) inhibited growth of the organism, and simultaneous treatment with 20 mM EDTA and 500 IU ml⁻¹ nisin was most effective.

MECHANISM OF ACTION

Microbial inhibition by EDTA is attributed to binding of divalent cations found in the bacterial cell wall. This increases cell permeability and thereby facilitates the action of antibacterial agents or degradative enzymes (Leive, 1968), releasing lipopolysaccharides from the outer membrane (Leive, 1965) or causing lysis (Eagon and Carson, 1965). Gray and Wilkinson (1965) also theorized that EDTA was active against *P. aeruginosa* through a mechanism involving chelation. Divalent metal cations, such as calcium and magnesium, were reported to function as a salt bridge in Gram-negative organisms, binding polysaccharides on the surface of the cell wall. Formation of EDTA complexes with these cations caused leakage of cell solutes and loss of viability. Bactericidal activity decreased as the pH was reduced, supporting chelation as the mode of action.

Studies of germination and growth from spores of toxigenic *B. cereus* in BHI showed reduced growth in the presence of less than 300 ppm EDTA (Bulgarelli and Shelef, 1985). EDTA did not affect the percentage of spore germination or release of calcium following heat activation and subsequent incubation. Reversal of this inhibition occurred by adding iron, zinc, or calcium at 500 ppm, or 1.49 mM. Magnesium was less effective.

Kraniak and Shelef (1988) studied the mechanism by which EDTA and its salts affected *S. aureus*. EDTA or its sodium or potassium salts in the range of 0.8 to 1.7 mM inhibited cell growth in BHI broth, but the calcium or iron salts were ineffective. Addition of Fe³⁺, Zn²⁺, or Ca²⁺ to EDTA-containing broth eliminated the inhibition at an EDTA/cation molar ratio of 1:1. In contrast, the effective Mg²⁺ concentration was seven times higher. This was explained by the low stability constant for EDTA/Mg²⁺ compared with that for the other cations, which makes Mg²⁺ less competitive in replacing Fe³⁺, Zn²⁺, or Ca²⁺ in the EDTA-cation complexes.

EDTA acts as a synergist for other antibacterial compounds. It has been reported to potentiate the activity of lysozyme, a mucolytic enzyme, by chelating divalent cations of the lipoprotein and lipopolysaccharide layers of the cell wall, thereby facilitating lysozyme access to the mucopeptide layer, where hydrolysis of the (1-4) glucosidic linkages occurs. Formation of spheroplasts, which are subject to osmotic lysis in a hypertonic environment, was reported by Moustafa and Collins (1969). Enhanced antibacterial activity of hen egg white lysozyme against *L. monocytogenes* in the presence of EDTA was reported in certain vegetables (Hughey et al., 1989).

EDTA alone does not inhibit botulinal outgrowth in cured meats but acts synergistically with nitrite in these products, evidently by sequestering a cation essential for the repair of nitrite-injured cells (Tompkin et al., 1978). With regard to mesophilic spores, acid blanching, followed by addition of EDTA to the can brine, was shown to control germination after sublethal thermal processes. The mechanism of inhibition using *C. sporogenes* PA 3679 appeared to involve collapse of the cortex peptidoglycan layers, possible hydration of the core, and a concomitant increase in the core volume (Okereke et al., 1990). The restoration of germicidal activity to QAC-resistant cells by EDTA was explained by disruption of a lipid-containing barrier surrounding the cell, which increases permeability and facilitates entrance of the sanitizing agents (MacGregor and Elliker, 1958).

APPLICATIONS

Effects of EDTA on foodborne organisms include studies in culture media and foods. Moustafa and Collins (1969) found EDTA to be inhibitory to *Pseudomonas fragi* in broth, but not in skim milk or half-and-half, because of the excess of metal ions in the dairy products. Studies in meats conducted by Tompkin and coworkers (1978) focused on the antibotulinal efficacy of combinations of nitrite, isoascorbate, and EDTA in perishable canned products. The addition of EDTA caused delay of botulinal outgrowth in pork heart meat in the presence of nitrite and ascorbate, whereas a control without EDTA showed no inhibition. In cured pork hearts, where levels of iron were sufficiently high, normal levels of nitrite and isoascorbate did not retard botulinal outgrowth. With EDTA, inhibition was restored. Neither ascorbate nor EDTA alone could delay botulinal outgrowth; however, both compounds shared the property of being able to act synergistically with nitrite to delay the growth of *C. botulinum*. In another study, EDTA and nitrite, with or without isoascorbate, provided potent inhibition. The effect of nitrite was enhanced to a greater degree by 500 μg EDTA g^{-1} than by 200 μg isoascorbate g^{-1} (Tompkin et al., 1979).

Studies in fish showed EDTA salts to be effective as dipping solutions for extending the shelf life of raw fish (Levin, 1967; Boyd and Southcott, 1968; Power et al., 1968). Levin (1967) found that an application of a 1% Na_4EDTA dip to haddock fillets for 1 minute failed to suppress the increase in bacterial numbers of fillets stored at 3°C compared to untreated controls. In odor and taste evaluations, however, shelf life was extended from 5 to 9 to 10 days. Percentages of *P. fluorescens* and *Pseudomonas putrefaciens* in the total spoilage flora of EDTA-dipped and nondipped fillets were not different. Bacterial growth without rapid spoilage was believed to occur as a result of inhibition of exocellular bacterial spoilage enzymes by the EDTA binding of required cations or by the binding of specific tissue substrates of spoilage enzymes. Exocellular enzymes of *P. putrefaciens* were found to be sensitive to EDTA.

A 1% EDTA solution used as a dip extended the shelf life of petrale sole and ocean perch fillets by repressing growth of *Pseudomonas* species. The sodium salts were more effective than the sodium-calcium salt, extending the shelf life at 0.5°C by 7 to 10 days (Pelroy and Seman, 1969). Kuusi and Loytomaki (1972) also reported that EDTA increased the shelf life of fish. In this study, rainbow trout and Baltic herring were dipped in 1% Na_2EDTA and then stored at 4°C. Control trout samples spoiled after 18 to 20 days, but the EDTA-treated samples were free of off-odors for the test period. Volatile basic nitrogen, trimethylamine, and hypoxanthine were also lower in the EDTA-treated fish. Differences between the EDTA-treated Baltic herring and the controls were less dramatic. More recently, treatment of fresh sardines, mackerel, and prawn with 0.1% and 1% Na_2EDTA dips for 10 minutes followed by storage on ice for 20 to 30 days resulted in reduced populations of *Pseudomonas* species (Surendran and Gopakumar, 1982).

With regard to *Clostridium* species, inhibition of germination and outgrowth of spores of *C. botulinum* type A in fish homogenates was reported at concentrations above 2.5 mM, and a greater effectiveness was attained at high pH values (Winarno et al., 1971).

Using the “preservative system” approach, Kabara (1981) proposed the use of EDTA in systems consisting of a number of compounds (e.g., monolaurin, EDTA, and BHA) in which EDTA functions by rendering the microorganisms more susceptible to the action of the other components of the system. Similarly, EDTA is used in applications of the “hurdle technology” approach aimed at inactivation of organisms in foods by treatment with a series of preservative factors, or hurdles (Leistner et al., 1995).

The use of EDTA in combination with lysozyme has been suggested as a preservative in foods because the latter is frequently found in human and animal cells. The Eisai Company patented a process for preservation of meat and fish products (Eisai Co., Ltd., 1966), and Igarashi and Zama (1972) patented a method for preservation of seafoods. Growth inhibition of the predominantly Gram-negative shrimp microbiota in 2% shrimp homogenate required 0.5% Na_2EDTA , whereas

0.02% Na₂EDTA was effective in combination with 50 µg/ml of lysozyme (Chander and Lewis, 1980).

Teotia and Miller (1975) studied the effect of lysozyme and EDTA treatment on recovery of *Salmonella* from turkey parts, and Samuelson et al. (1985) studied the effect using broiler legs. The former reported no recovery of *Salmonella* Senftenberg after treatment with 0.1% lysozyme plus 0.5% EDTA; the latter observed a reduction in the numbers of *Salmonella* Typhimurium but no inhibitory effects using *Salmonella* Heidelberg and the lysozyme-EDTA dip.

Combinations of EDTA with lysozyme, nisin, and other antimicrobials or their combinations in packaging films are other examples of enhanced antimicrobial effects in packaged foods (Padgett et al., 1998; Natrajan and Sheldon, 2000; Hoffman et al., 2001; Cutter et al., 2001).

TOXICOLOGY

EDTA decreases the gastrointestinal absorption of a number of toxic agents by increasing the permeability of the membrane through chelation and removal of calcium (Klaassen, 1980). The calcium disodium salt is used in the treatment of lead poisoning. The Food and Agriculture Organization/World Health Organization acceptable daily intake for EDTA is 0 to 2.5 mg/kg body weight at a 0.01% level of use, with levels not exceeding 1.25 mg/kg body recommended. The compound exhibits a low order of animal toxicity. The LD₅₀ in rabbits has been determined as 47 mg/kg when given intravenously and 2.3 g/kg when fed orally. Human patients given infusions of disodium EDTA over a 2-year period showed no serious side effects, and a bioassay for EDTA carcinogenicity proved negative (Kabara, 1981). Because the disodium salt can interfere with mineral bioavailability, its use as a food additive is not recommended unless for the purpose of chelating calcium ions, such that no excess of the Na₂EDTA remains.

REGULATORY STATUS

EDTA is approved as a food additive for direct addition to food in the form of calcium disodium EDTA (21 CFR 172.120) and disodium EDTA (21 CFR 172.135) (CFR, 1990). Designated primarily as a preservative and to promote color retention and inhibit discoloration, it is approved for use in a variety of foods, including salad dressing, mayonnaise, cooked canned crab meat, and sausage. Concentrations, calculated as anhydrous calcium disodium EDTA, are 25 to 500 ppm, depending on the food.

ASSAY

EDTA is readily determined as its alkali metal salts in the absence of alkaline earth metals and heavy metals by titration with standardized calcium acetate solution. Sodium oxalate is used as an internal indicator. The end point is the appearance of permanent turbidity. Several methods have been developed to determine the total amount of EDTA at low concentrations in the presence of certain metals sequestered by it.

Nickel may be used to displace calcium and magnesium from EDTA because it is preferentially complexed. A gravimetric method has been developed in which a known amount of nickel is added. Excess unsequestered nickel is precipitated as the hydroxide and converted to the dimethylglyoxime salt for gravimetric determination. The difference, representing sequestered nickel, is equivalent to the amount of EDTA present.

Darbey (1952) developed a colorimetric procedure whereby total EDTA in low concentrations could be quantified in the presence of certain metals. Nickel was used to displace calcium and magnesium from the complex with EDTA, and the excess was removed. The nickel chelate formed was subjected to dissociation in strong acid solution to liberate the sequestered metal. A blue-red color was formed with the addition of potassium dithioxalate. This procedure was found to be sensitive to 0.2- to 0.5-mg EDTA in a 100-ml sample solution.

SODIUM BICARBONATE

Sodium bicarbonate is a general-purpose or multiple-purpose GRAS food compound. The use of the compound as the source of carbon dioxide for leavening of baked goods and for control of pH, taste, and texture is well established. Reports on the antimicrobial effects of this common food additive first appeared in the 1980s. It should be noted that unlike most indirect antimicrobials discussed in this chapter, the evidence for the antimicrobial effects of bicarbonate has originated from intentional addition to foods that normally do not contain this food additive.

CHEMISTRY

Sodium bicarbonate is a salt of a strong base (sodium hydroxide) and a weak acid (carbonic), prepared from sodium carbonate, water, and carbon dioxide (Lowenheim and Moran, 1975). It is alkaline, and its ionization in solution frees the cation and the bicarbonate (HCO_3^-), which react with acids, resulting in formation of new salts and carbonic acid. The latter dissociates to yield carbon dioxide and water. Carbon dioxide, which is liberated progressively as the temperature increases, is a common leavening gas in baking. The molecular weight (MW) is 84.00, and the pH of 0.1 M aqueous solution at 25°C is 8.3. The ammonium and potassium salts (MW, 79.06 and 100.11, respectively) are also used in baking powder.

ANTIMICROBIAL ACTIVITY

There are limited studies on the antimicrobial effects of sodium bicarbonate. Inhibitory effects were first reported against oral bacteria (Miyasaki et al., 1986). The potential use of the compound as an antifungal agent was examined by Montville and Goldstein (1987), who showed that incorporation of bicarbonate into Czapek's agar reduced *A. parasiticus* cell viability. Sodium bicarbonate inhibited growth of a number of food-related bacteria, yeast, and mycotoxigenic fungi in model systems (Corral et al., 1988; Depasquale et al., 1990). Ammonium and potassium salts were also inhibitory. *In vitro* inhibitory effects of bicarbonate on growth of fungi were also demonstrated by Williams et al. (1997).

APPLICATIONS

In a study by Montville and Goldstein (1989), the ability of sodium bicarbonate and ammonium bicarbonate to inhibit aflatoxigenesis in corn was investigated, and a method for applying the compound evenly to corn was developed. Using an inoculum of *A. parasiticus* spores, aflatoxin levels in samples treated with 0.17% sodium bicarbonate were reduced to one third those of untreated samples after 3 weeks at 30°C. The practicality and economics of bicarbonate applications to grains as an antifungal agent have not yet been studied. Ammonium and sodium bicarbonate were found to inhibit spoilage of apple juice by *Hansenula wingei* and *S. cerevisiae*, but off-flavors and darkening of the juice were seen at effective concentrations (Curran and Montville, 1989).

Dipping cod fillets in ammonium or sodium bicarbonate solution was reported to reduce microbial growth after 8 days at 4°C (Curran et al., 1990). Total aerobes, proteolytic bacteria, and H_2S -producing bacteria were inhibited. Mixtures of ammonium and sodium salts, which were also effective, resulted in reduction of the ammonia odor. All bicarbonate treatments increased the fish pH from 7.0 to 8.2 to 8.5, thereby improving texture and moisture retention. Sensory evaluation indicated that bicarbonate treatment resulted in an inferior product. The effect of various carbonate sources on the survival of *E. coli* in dairy cattle manure was reported in 2001 (Arthurs et al., 2001). Unlike the carbonates (Na and K), the bicarbonate at concentrations of 16 g/kg manure slurries did not decrease the numbers of the organism.

MECHANISM OF ACTION

Studies in Czapek's agar showed that both sodium and potassium bicarbonate inhibited growth of *A. parasiticus* at an initial medium pH of 7.5 but were ineffective when the initial pH was 5.5 (Montville and Goldstein, 1987). Because growth was not inhibited at a pH as high as 10.5 *per se*, the authors concluded that inhibition could not be attributed to pH elevation but was the result of bicarbonate ion (dominant form at pH 7.5) rather than of carbon dioxide (dominant form at pH 5.5) (Corral et al., 1988; Montville and Goldstein, 1987). Likewise, growth inhibition of *A. parasiticus*, *H. wingei*, and *S. cerevisiae* in other studies could not be attributed to pH elevation alone and appeared to be associated with the bicarbonate ion (Curran and Montville, 1989; Depasquale et al., 1990).

Curran et al. (1990) noted that the increase in pH associated with the use of bicarbonate may affect the overall microbial ecology of highly acid foods and may lead to spoilage by atypical organisms. They suggested that high-pH foods with a buffering capacity (e.g., fish) may be more suitable for evaluation of the inhibitory effects by bicarbonate.

Montville and Goldstein (1987) observed that *A. parasiticus* growth in the presence of 1% sodium bicarbonate was accompanied by the production of unusual pigments, suggesting accumulation of precursors in the aflatoxin biosynthetic pathway. El-Nabarawy et al. (1989) extracted *A. parasiticus* NRRL 2999 cultures grown in the presence of bicarbonate. Using thin-layer chromatography, accumulation of averufin and vericolorin A was observed, and this was confirmed by desorption chemical ionization mass spectrometry. These data suggested the possibility that bicarbonate may inhibit enzymes involved in the conversion of these intermediates to sterigmatocystin and ultimately to aflatoxin.

REGULATORY STATUS

The sodium, potassium, and ammonium bicarbonates are GRAS and lack toxicity (21 CFR 582.1613 and 582.1721). Sodium, potassium, or ammonium bicarbonate is used in food at levels up to 2% (CFR, 1990).

ASSAY

Sodium bicarbonate is assayed by dissolving in water and titrating with 1 N HCl until the solution becomes faintly pink using methyl red as indicator. The solution is then heated to boiling, cooled, and titrated until the pink color no longer fades after boiling. Each ml of 1 N HCl is equivalent to 84.01 mg NaHCO₃ (National Academy of Sciences, 1983).

ETHANOL

Ethanol or ethyl alcohol (EtOH, C₂H₅OH) has been used as an antimicrobial since the first alcoholic fermentation was practiced to preserve fruit.

PHYSICAL AND CHEMICAL PROPERTIES

EtOH is obtained by fermentation of sugar. It is a colorless liquid, miscible in water, with an MW of 46.07 and boiling point of 78°C.

ANTIMICROBIAL ACTIVITY AND MECHANISM OF ACTION

EtOH is bactericidal but is not sporicidal. It has poor penetrating power and is inactivated by organic matter (Banwart, 1989). At high concentrations the activity against microorganisms is by denaturing proteins in the cytoplasm. The compound is bactericidal at high concentrations (60% to 75%). At

lower concentrations (5% to 20%) it may exert inhibitory effects in combination with other preservatives (Banwart, 1989) and by lowering the water activity of foods (Lueck, 1980).

APPLICATIONS

EtOH is used as a solvent in flavoring extracts (e.g., vanilla). Fortification of wines to a total EtOH content of around 20% is required for microbiological stability. Examples of such products are sweet wines, such as port and sherry. Shapero et al. (1978) reported inhibition of *S. aureus* by 2% to 4% EtOH in an intermediate-moisture food. The effect of EtOH vapor on growth of *S. cerevisiae* in gas-packaged baked products was investigated by Smith et al. (1987). EtOH vapor incorporated into packaged apple turnovers completely suppressed growth of the yeast during a 21-day storage period at ambient temperature, whereas turnovers packaged in air or CO₂/N₂ (60:40) were swollen after 14 days of storage because of growth of *S. cerevisiae*. The shelf life of Chinese cabbage salted with 2% to 3% NaCl was extended 3 to 5 days by the addition of 3% EtOH with heating for 2 hours at 50°C. Growth of Gram-negative bacteria was inhibited, but no effect was seen on *B. subtilis* or *Staphylococcus* species. The effectiveness of EtOH additions was demonstrated with other vegetables. Mold growth was inhibited in salted bitter melon by 10% EtOH, and the shelf life of Chinese noodles was extended to 2 to 4 days by the addition of 1.5% EtOH with 0.75% propylene glycol (Miyao, 1989).

The preservative effect of EtOH in an egnog-like product was studied by Notermans et al. (1990). The drink, consisting of whole egg, sucrose (25%), and EtOH (7%), was artificially contaminated with *Salmonella* Enteritidis, *S. Typhimurium*, *S. aureus* (three strains), *B. cereus*, and *L. monocytogenes*, and growth was determined during storage at 22°C and 5°C. The decrease in the pathogenic microorganisms was much slower at the lower temperature, but total colony counts increased at the higher temperature, resulting in a limited shelf life. It was concluded that a public health risk may still exist from the product with a low EtOH content. EtOH is approved for use as a direct antimicrobial, for example, on pizza crusts before final baking at levels not to exceed 2% by product weight (CFR, 1990).

TOXICOLOGY

The LD₅₀ of EtOH for the rat is 13.7 g/kg body weight following oral administration (Spector, 1956). In humans, levels of 4 to 6 g/L blood can be hazardous, depending on tolerance of the individual, which increases with chronic consumption (Lueck, 1980).

REGULATORY STATUS

EtOH is a direct food substance approved as GRAS (21 CFR 184.1293). It is a component of a large number of foods, whether formed naturally in them or added.

ASSAY

EtOH is often determined in foods by distillation, collection of the distillate, and determination of the alcohol by specific gravity measurements using a pycnometer. Gas chromatography is used for EtOH determination in beer and in canned salmon (AOAC, 1990), and low concentrations can be determined enzymatically using alcohol dehydrogenase.

PROPYLENE GLYCOL

CHEMISTRY AND PRODUCTION

Propylene glycol ($\text{CH}_3\text{-CHOH-CH}_2\text{OH}$), also known as 1,2-propanediol, has some antimicrobial activity because of its reduction of water activity. It serves the following functions in foods: anticaking agent, antioxidant, dough strengthener, emulsifier, flavor agent, humectant, processing aid, solvent and vehicle, stabilizer and thickener, surface-active agent, and texturizer (21 CFR 184.1666) (CFR, 1990). It does not naturally occur in nature and is manufactured by treating propylene with chlorinated water to form the chlorohydrin, which is converted to the glycol by treatment with sodium carbonate solution.

ANTIMICROBIAL ACTIVITY AND MECHANISM OF ACTION

When semimoist pet foods were introduced, propylene glycol was the most common humectant-antimicrobial agent used (Acott et al., 1976). Acott et al. (1976) investigated the effect of propylene glycol on the growth of *Aspergillus niger*, *Aspergillus glaucus*, and *Staphylococcus epidermidis* in semimoist dog food stored at 23°C. At 0.85 a_w , 5% propylene glycol at pH 5.4 was effective at controlling growth. When the a_w was raised to 0.88, propylene glycol controlled growth in combination with 0.3% calcium propionate or 0.3% potassium sorbate. Propylene glycol at 6.2% in combination with 31 ppm NaNO_2 and 9.1% propylene glycol in combination with 24 ppm NaNO_2 were effective inhibitors of *C. botulinum* (Hall and Maurer, 1986). At 37°C, 5% or more propylene glycol inhibited the growth of *S. Typhimurium* in 0.1% reconstituted nonfat dry milk. At concentrations >20%, $a_w < 0.96$, no recovery of *S. Typhimurium* was possible after 4 hours (Airoldi and Zottola, 1989). Kondaiah et al. (1985) investigated the efficacy of chemical dips in controlling growth of *E. coli*, *S. aureus*, *Streptococcus faecalis*, and *C. perfringens* on unchilled fresh beef. The addition of propylene glycol to mixtures of potassium sorbate, sodium acetate, and sodium chloride did not enhance inhibition. The following mechanism of action for propylene glycol was proposed by Herman et al. (1980): The activity may be related to a tendency of propylene glycol molecules to orient themselves between lipid and water phases of the microbial membrane, which possibly alters the permeability of the cell membrane and thus interferes with membrane function.

TOXICOLOGY

Propylene glycol is noncarcinogenic and until recently was believed to have a very low LD_{50} in the region of 20 to 30 g/kg body weight (Lueck, 1980). Low doses are still considered innocuous, but higher doses can cause depression of the central nervous system, on which the compound has a narcotic effect about one third that of ethanol (Christopher et al., 1990). Most cases of human toxicity have occurred during use of propylene glycol-containing drugs or ointments, especially when renal or hepatic insufficiency is present. In cases of toxicity the reported doses have ranged from 0.057 to 9.0 g/kg body weight per day, depending on the study and the type of drug or ointment administered to the patients (Christopher et al., 1990).

REGULATORY STATUS

Propylene glycol is classified as GRAS and can be used in foods in levels not to exceed current good manufacturing practice. Current maximum levels include 5% for alcoholic beverages, 24% for confections and frostings, 2.5% for frozen dairy products, 97% for seasonings and flavorings, 5% for nuts and nut products, and 2% for all other food categories (21 CFR 184.1666).

DIACETYL

Diacetyl, 2,3-butanedione, is best known for producing the characteristic aroma and flavor of butter (Van Neil et al., 1929). In addition to other dairy products, it is found in red and white wines, brandy, roasted coffee, ensilage, and other fermented foods. Some strains of *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* produce the compound (Jay, 1982a). Typically, diacetyl is a greenish-yellow liquid with a boiling point of 88°C and the sharp odor of butter (Jay, 1982a). Diacetyl is GRAS for use as a flavoring in foods (21 CFR 184.1278) (CFR, 1990).

ANTIMICROBIAL ACTIVITY

Jay (1982b) screened 70 organisms, including lactic acid bacteria, nonlactic Gram-positive bacteria, yeasts, molds, pseudomonads, and nonpseudomonad Gram-negative bacteria, on plate count agar at pH 5.0 to 8.0 in the presence of varying concentrations of diacetyl. The lactic acid bacteria were the most resistant, and the pseudomonads were the most sensitive. The compound was most active below pH 7.0. Excluding the lactic acid bacteria, 300 ppm diacetyl completely inhibited 90% of the strains tested. Some pseudomonads were inhibited by 86 ppm at pH 6.0. Diacetyl was also effective at 400 ppm in inhibiting the growth of spoilage bacteria over an 8-day period in refrigerated, freshly ground beef (Jay, 1982b). Skoog and Tatini (1990) investigated the effect of diacetyl in conjunction with heat on the growth and survival of *L. monocytogenes*. Increased cell injury and death were reported when cells were heated with 1000 ppm diacetyl in trypticase soy broth with yeast extract. *E. coli*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, and *Salmonella anatum* were inhibited by diacetyl (344 ppm), but *Listeria* organisms, including *L. monocytogenes*, *L. innocua*, and *L. ivanovii*, showed very low loss of viability (Motlagh et al., 1991). Tests with *Vibrio vulnificus* showed that diacetyl at 50 ppm was lethal within 24 hours to both opaque and translucent morphotypes of the organism (Sun and Oliver, 1994). A similar concentration of diacetyl also inhibited *E. coli* O157:H7 and *S. Typhimurium* in laboratory medium (Kang and Fung, 1999). Tested during meat fermentation by *Pediococcus acidilactici*, a 1-log CFU/g decrease in numbers of each of these organisms was observed after 24 hours in samples containing 300 ppm diacetyl. Growth and acid production by *P. acidilactici* were not affected by diacetyl.

MECHANISM OF ACTION

Jay et al. (1983) postulate that diacetyl antagonizes arginine utilization by reacting with arginine binding sites on the proteins of Gram-negative bacteria. Gram-positive bacteria may be more resistant because of their lack of similar periplasmic binding proteins and their larger amino acid pools.

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18 Antibiotic Residues in Foods and Their Significance

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CONTENTS

Introduction	599
Scope of Chapter.....	600
Incidence of Residues	601
Sampling or Monitoring	601
Sampling Components	601
Survey Results.....	602
Animal Residue Data 1998.....	603
Nonviolative Samples 1998.....	604
Animal Residue Data 1999.....	606
Residues in Milk.....	609
Residues in Eggs.....	612
Residues in Fish.....	614
Antibiotics and Antimicrobials in the Aquatic Environment.....	615
Effects of Combinations of Compounds at Residue Levels on the Selection for Antibiotic Resistance in Bacteria.....	615
Antibiotic and Antimicrobial Resistance Selection Caused by Nutraceuticals	617
Overview	617
References	618

INTRODUCTION

There is little doubt that residues of antibiotics and antimicrobials will occur in foods of animal origin. It is not only that such residues will occur; it is both the frequency and the number of such residual compounds that is important. It is not surprising that residues of antibiotics, antimicrobials, growth promotants, anthelmintics, and pesticides are found in foods of animal origin. The amounts of those chemicals used in agriculture are difficult to ascertain accurately. The Animal Health Institute (AHI) estimated that the overall poundage of antimicrobials was 17.8 million pounds for both therapeutic and nontherapeutic uses in all animals. In contrast, the Union of Concerned Scientists (UCS) estimated antibiotic/antimicrobial usage at 24.6 million pounds for nontherapeutic purposes in three species only — poultry, hogs, and cattle — and has no estimate for therapeutic use in animals (Mellon et al., 2001). The argument over usage levels does not obviate the fact that large amounts of antibiotic and antimicrobial compounds are used in animal agriculture for a number of purposes: (1) nontherapeutic growth promotion, (2) disease prevention, and (3) disease treatment. The disease prevention and growth promotion uses, at times, seem to overlap and vary from a few grams to 200 g/ton. Levels over 200 g/ton are usually used for treatment of disease. With the millions of pounds of antibiotics/antimicrobials used in animals produced for

food for human consumption, it is almost a certainty that antibiotic/antimicrobial residues will occur in food products of animal origin. Residues will occur from abuse of withdrawal times, mistaken as well as deliberate, misuse of drugs, and the biological variability in animals held to the proper withdrawal times. It should be emphasized that the vast majority of residues will occur from the misuse of medications. The most important aspect is to minimize antibiotic/antimicrobial residues in food to remove the burden of residues from the arena of chemical insults to which the human being is exposed.

The question of what constitutes a residue is one that usually engenders some debate. These authors define a residue as any amount of drug, antibiotic, or antimicrobial, including biologically active metabolites, which can be present and hopefully measured in the product. This definition corresponds reasonably to that espoused by the Food and Drug Administration/Center for Veterinary Medicine (FDA/CVM). Thus, there are two major types of residues: those that fall below the tolerance level or maximum residue level (MRL) and those that are violative, greater than the tolerance level or MRL. The designation "safe level," used commonly for antibiotic/antimicrobial residues in milk, may or may not be the official tolerance level. There is also the second previously silent residue from antibiotic usage, at least until the recent years, namely, the residues of antibiotic/antimicrobial-resistant microorganisms on foods. This second residue will not be considered in any detail except to say the presence of the resistant microorganisms currently has taken precedence over the presence of antibiotic/antimicrobial residues. The rationale is very simple: the extremely small number of adverse idiosyncratic responses resulting in severe allergy or death related to antibiotic/antimicrobial residues versus the estimated 76 million cases of food-related illnesses and 5000 estimated deaths (Vogt, 2000).

The question of what constitutes a food should be added to the mix of the discussion. Foods are usually considered as edible products of plants, animals, and aquatic species and include fruits, vegetables, muscle meats, and edible organs from beef, swine poultry, sheep, goats, horses, eggs, milk, milk products, fish, and crustaceans. In addition, these authors look at water as essential both to sustain life and health. Although water may or may not contain essential nutrients, outside of electrolytes, it is essential in processing many foods and as an ingredient in many products. One fact that should never be forgotten or even underestimated is that the final repository for drugs taken by humans and to some extent animals is our aquatic systems. The eventual reuse of such waters for potable, domestic, and industrial purposes and the probability of potable water containing low level residues is very real.

SCOPE OF CHAPTER

This chapter differs in scope from an earlier chapter on this subject; there will be only modest reference to the older residue data. Fortunately, the 1993 chapter in *Antimicrobials in Foods*, 2nd edition (P.M. Davidson and A.L. Branen, Eds., Marcel Dekker, Inc., 1993, pp. 571–595) was reprinted in *Food Biotechnology*, (14:147–171, 2000) (Katz and Brady, 1993, 2000). With the easier ability to reference this current journal, these authors felt that a reiteration of much of that chapter was unnecessary. Instead, this chapter will focus on the incidence of antibiotic/antimicrobial drug residues found in foods of animal origin as reported in the large-scale surveys performed by the Food Safety Inspection Service (FSIS)/U.S. Department of Agriculture (USDA), the statistical basis of the surveys, and data gleaned from literature reports of residues found in foods of animal origin, the residues of drugs found in water, and the implications of bacterial exposures to low levels of drugs. The authors hope to stimulate discussions concerning bacterial exposures to multiple residues and the development of bacterial antibiotic resistance. Areas such as the antimicrobial properties of some of the commonly used nutraceuticals and their ability to increase antibiotic/antimicrobial resistance in bacteria also will be introduced.

INCIDENCE OF RESIDUES

The incidence of residues in the food supply will always be a matter of argument in the context of the debate concerning the nutritional qualities of organic versus nonorganic foods. Incidence is somewhat a perception because it is impossible to guarantee that the food supply will contain no more than a small percentage of foods having residues greater than the tolerance level. The sampling procedures are like political polls, yielding only (at any given point in time) a probability that certain events or frequencies will occur within the defined limits of error. There are no absolutes. Hence the continuum of debates over the frequency of antibiotic/antimicrobial residues present in the food supply.

SAMPLING OR MONITORING

Like any assay for a given analyte, the analytical result will only be as good as the sample taken. The same situation holds true for the yearly, large-scale surveys performed by the FSIS. The basis of the surveys is strictly statistical and only has the ability to sample a relatively small percentage of the commercial food supply. The FSIS cautions that “care must be taken when making statistical inferences from these data.” The data are designed to detect at a predetermined statistical confidence level specific compounds in what is termed a “slaughter class” and not to provide an overall national violation incidence. The percentage or frequency of violation for each “slaughter class” is a statistically valid estimate based on the randomness sampling of the model. The data using a two-sided 95% confidence limit interval will give the probability of a sample of a specific slaughter class to fall between the upper and lower estimates for the population.

Sampling Components

There are four sampling components to the yearly National Residue Program (NRP) for domestically produced products (Domestic Residue Data Book, 1998). These are (1) the monitoring program, which takes a random sampling of specified healthy and normal animal species, at slaughter, to provide information on the frequency of residue violations; (2) special projects cover those cases not falling under the criteria set for the monitoring program, cases where there are no slaughter volume data, and cases where there is no basis of estimating violation frequencies or to develop information as to the incidence and levels of certain residues; (3) surveillance sampling covers those areas where residue problems exist and is designed to define the extent of the problem so that actions can be taken to reduce an abnormal frequency of the residues; and (4) enforcement is used primarily to follow producers having a record of marketing animals with violative residue levels and to legally correct the problems. The enforcement component also targets animals that do not appear healthy and normal and includes samples from the carcasses of animals that have been condemned. Carcasses sampled under the monitoring and special projects are not held pending the results. The purpose is to provide information concerning the emergence of an area wherein residues are becoming a problem. All of these components are part of the Hazard Analysis Critical Control Point (HACCP) responsibilities of the FSIS.

The planned prevalence or frequency sampling occurs at 4 levels, 460 samples/year, 300 samples/year, 230 samples/year, or 90 samples/year. The 300 sample/year frequency provides a 95% confidence level that violative sample occurring in 1% of the slaughter class could be detected or that greater than 1% violation frequency will be found 95% of the time. This rests on the premise that normal-appearing, inspected, and passed carcasses will be a sufficiently homogenous sampling that will be indicative of the national animal food supply.

The import residue program related to meat, poultry, and egg products focuses on the equivalence of the exporting country's inspection system, the certification of specific facilities, and the periodic FSIS inspection of these facilities for export eligibility. Part of the program also includes

TABLE 18.1
Number of Samples Required to Ensure Detection of a Residue Problem
Affecting a Given Percentage of the Sampled Population

Percentage Violative in Sample Population	Probability of Detection (Percent)			
	90	95	99	99.9
	Samples Required			
10	22	29	44	66
5	45	59	90	135
1	230	299	459	688
0.5	460	598	919	1379
0.1	2303	2995	4603	6905
0.05	4605	5990	9209	13,813

reinspection at the port of entry to maintain the effectiveness of the inspection program. The equivalent residue-control program consists of the random sampling of animals at slaughter, using assay systems that are validated, assaying target tissue regardless of whether these tissues are exported, assaying for potential contaminants, and the random sampling of eggs to be used for processing. The FSIS relies on the country's inspection system to certify that all applicable standards are met and audits the foreign inspection system.

There are four screening assays used by the FSIS and all are defined as rapid on-site tests. These are (1) the Sulfa-on-Site (SOS), which measures swine urine for sulfonamide residues; (2) the Calf Antibiotic and Sulfonamide Test (CAST), which is used on the kidney or liver tissue of bob veal calves (<3 weeks of age and less than 150 lbs.); (3) the Swab Test on Premises (STOP), which assays for antibiotic residues in the kidney tissue of all production classes of animals (cattle, swine, chickens, turkeys, and sheep); (4) the Fast Antimicrobial Screen Test (FAST) for antibiotic and sulfonamide residues, which is a swab test of kidney or liver tissues of cows and bob veal calves. A carcass that indicates a positive result is held until confirmatory laboratory assays are performed. If positive, the carcass is not allowed into the food supply. If the screening assay is negative, the carcass is allowed into commerce unless condemned for other regulatory reasons.

Table 18.1 shows the number of samples necessary to ensure detection of a residue problem that affects a given percentage of a samples population. This table basically follows a rational approach and indicates as the number of violative samples decreases (experience-based information) there will be a large increase in the number of samples required for detection. Where there are large numbers or a high percentage of residues expected, there will be the obvious much smaller number of samples required. At the 95% confidence limit, a minimum sampling of 299 animals should be sufficient to detect a violative frequency of 1%; if the violative frequency is 0.5%, the sample size doubles; if the frequency is 0.1%, the sample size is 10 times. If a higher probability than 95% is required or desired, the sampling of animals must increase significantly.

Survey Results

In reviewing the data for presentation, it became evident that summarizing the information accumulated over the last 10 years would not yield anything significant beyond the past 2 or so years. The most recent performance of the animal industry as monitored by the regulatory agencies is the most significant factor.

TABLE 18.2
Slaughter Classes and Production Numbers of Animals

Class	Numbers	Class	Numbers
Horses	68,783	Goats	455,076
Bulls	721,868	Market hogs	100,365,553
Beef/dairy cows	7,093,206	Boars/stags	735,616
Heifers	12,871,623	Sows	3,578,225
Steers	19,818,380	Chickens, young	7,556,363,108
Bob calves	690,506	Mature	164,353,454
Formula-fed calves	727,667	Turkeys, young	265,266,399
Nonformula-fed calves	24,592	Mature	1,871,937
Heavy calves	40,665	Ducks	23,050,277
Sheep	214,892	Rabbits	317,022
Lambs	3,556,435	Geese	142,428

ANIMAL RESIDUE DATA 1998

The FSIS (Domestic Residue Data Book, 1998) reported the 1998 production for the various "slaughter classes" included a total of 151.28 million head of livestock and 8.01 billion birds. The monitoring program sampled seven classes of animal drugs and pesticides, performing 26,888 analyses, which included antibiotics, antimicrobials, chlorinated hydrocarbon and organophosphate insecticides, arsenicals, antihelmintics, and other drugs. Of the 7829 samples assayed for antibiotics, only 38 violations were found in 37 animals of all slaughter classes. Horses were responsible for 21 of the antibiotic violations. Although cautioned against by the FSIS (using totals of all slaughter classes), these violations represent a violative sample frequency of 0.48%. If the violative horse samples are removed (because in the United States, horsemeat is not usually sold for human consumption), there remain but 17 violative samples or a frequency of 0.21% across all slaughter classes, a very low incidence. Table 18.2 shows the slaughter classes organized by the FSIS and the production numbers slaughtered. Of course, it is a given that these frequencies are related only to federally inspected plants.

The on-site screening assay results also indicated a relatively low incidence of violative samples. Using the CAST system, 8958 analyses were performed on bob veal calves resulting in 82 violative animals or 0.92% of the slaughter class. In contrast, in 1997 (Domestic Residue Data Book, 1997), 11,988 animals were sampled finding violative residues in 55 animals or a 0.46% violative incidence. There should not be any misinterpretation of such relatively modest increases or decreases. In both years, the incidence was low.

A total of 37,633 STOP analyses were performed on samples taken from slaughter classes of horses, cattle (really undefined), sheep/lambs, goats, swine (undefined), and even ostriches. Of these samples there were 220 violative samples or a violative frequency of 0.58%. In 1997, 33,709 samples were assayed; 150 of those animals assayed were violative, an incidence of 0.44%. Again, the overall incidence in these slaughter groups was relatively low and consistent with the previous year.

The greatest number of assays was performed using the FAST system; 108,020 assays were performed on cattle, sheep, goats, swine, and other animals. Of this relatively large number, 751 or 0.70% were indicative of violative residues. In 1997, 109,021 animals were sampled with 472 being found violative, a 0.43% incidence.

The results of the SOS sampling of 11,109 animals were 28 violative animals or a frequency of 0.25%; in 1997, there were 10,072 animals sampled with 9 violative, an incidence of 0.09%.

TABLE 18.3
The 95% Confidence Intervals of Monitoring Samples for Antibiotics

Slaughter Class	Percentage Violative	95% Confidence Interval
Horses	4.5	2.8–6.9
Bulls	0	0.0–1.5
Beef cows	0	0.0–0.8
Dairy cows	0.4	0.0–1.5
Heifers	0.3	0.0–1.8
Steers	0.2	0.0–1.2
Bob calves	0.5	0.1–1.8
Formula-fed calves	0.8	0.2–2.0
Nonformula-fed calves	1.2	0.2–3.4
Heavy calves	1	0.2–3.0
Sheep	0	0.0–1.2
Lambs	0	0.0–1.1
Goats	0	0.0–1.1
Market hogs	0	0.0–0.8
Boars/stags	0.5	0.0–2.5
Sows	0	0.0–0.7
Young chickens	0	0.0–0.9
Mature chickens	0	0.0–1.6
Young turkeys	0	0.0–0.8
Mature turkeys	0	0.0–2.3
Ducks	0	0.0–0.7

Table 18.3 presents a probability approach to the occurrence of violative residues in the various slaughter classes. Regardless of the low frequency of violative residues being found, there is a finite potential of residues occurring in all slaughter classes, and these data show the ranges of these possibilities.

The probabilities listed are at best “guesstimates” of what the odds would be of residues occurring in a given slaughter class. There are no definitive numbers on the frequency of occurrence of violative samples in any given slaughter class; there are only ranges of potential occurrence. Statistical probabilities are the best that can be achieved because no one organization or a number of organizations can sample and analyze sufficient numbers of animals to allow for greater accuracy in estimating violative frequency. From a practical point of view there is also insufficient budget, personnel, laboratory capacity, and data-handling capacity to accomplish much more. These estimates do allow analysts to find problem areas and establish procedures and resources for surveillance.

The differences in the intervals when the percentage of animals sampled is 0.0 are a function of the number of animals sampled. Statistically, the ranges remain low. In fact, the 1998 violative frequencies for antibiotics were essentially the same as were found in 1989 (Katz and Brady, 1993), comparing the actual percentages of the samples that were violative. We believe that the monitoring samples yield the best insight as to the real incidences in the marketplace. Obviously, violative frequencies in the enforcement samples should always be higher because the sampling occurs in areas that have a previous history of residue problems or abuse of drug usage.

NONVIOLATIVE SAMPLES 1998

The picture of the frequency or incidence of violative residues occurring in monitoring samples is similar to that found for the nonviolative (NV) samples or samples that have residues but fall below

TABLE 18.4
Frequencies of Nonviolative Residues of Antibiotic and Sulfonamides in the Various Slaughter Classes of Animals

Slaughter Classes	Antibiotics			Sulfonamides		
	Samples	Positives	Percentage	Samples	Positives	Percentage
Horses	442	0	0.00	226	0	0.00
Cattle						
Bulls	244	0	0.00	247	2	0.81
Beef Cows	464	1	0.22	306	0	0.00
Dairy Cows	479	0	0.00	310	0	0.00
Heifers	299	0	0.00	234	0	0.00
Steers	479	0	0.00	321	0	0.00
Calves						
Bob	410	15	3.66	407	1	0.25
Formula-fed	510	35	6.86	372	2	0.54
Nonformula-fed	256	4	1.56	258	0	0.00
Heavy	286	4	1.39	223	1	0.45
Sheep	294	0	0.00	93	0	0.00
Lambs	348	1	0.29	103	0	0.00
Hogs						
Market	463	34	7.34	485	4	0.82
Boars/Stags	220	0	0.00	217	2	0.92
Chickens						
Young	429	1	0.23	278	1	0.36
Mature	234	0	0.00	233	2	0.86
Turkeys						
Young	468	12	2.56	307	3	0.98
Ducks	525	4	0.76	268	0	0.00

the MRL or tolerance levels. Table 18.4 shows the incidence of positive-confirmed NV residues of antibiotics and sulfonamides in the various slaughter classes. Again, it should be noted that the overall incidences of residues are relatively low, with the exceptions of calves and market hogs, and those residues are all legal.

Combining categories is what the FSIS never intended for these data. However, we feel that by combining obvious categories such as the combining of all NV residues found in cattle, calves, sheep, swine, chickens, turkeys, and ducks gives a clearer picture of where some potential problems may be residing. The incidence of NV antibiotic residues in cattle was 0.05%, and for sulfonamides it was 0.12%; in calves, the NV antibiotic residue incidence was 3.96%, and for sulfonamide residues it was 0.32%; in sheep the overall NV residue frequency picture is very low, 0.16% for antibiotics and 0.0% for sulfonamides. In contrast, the NV antibiotic residue frequency for swine was 4.76%, and for sulfonamides it was 0.78%; for chickens, the NV antibiotic residue incidence was 0.61%, and it was 1.59% for sulfonamides. In turkeys, the incidence of NV antibiotics is 2.54%, and for sulfonamides it was 0.64%. This is a reversal of levels of NV residues in two species of poultry and really has no direct explanation. The third species of poultry, ducks, had low frequencies of residues, NV antibiotics being 0.76% and sulfonamides being 0.0%. If any information is to be gleaned from these data, it is the relatively higher incidence in swine and calves, which is not unexpected from current feeding practices. Perhaps the frequency of sampling these categories should be somewhat higher because these values may indicate some “hot” spots. Regardless, the frequency of NV residues of both antibiotics and sulfonamides is low, below 5% in the animals surveyed, and all are legal residues.

If one looks at these data in a general fashion, it appears that at least some 95% of animals most commonly used for food in the United States are free of any antibiotic and sulfonamide residues. At the same time, one is cautioned not to overestimate the safety of these foods because the samples taken represent but a very small fraction of the animals used in the food supply.

ANIMAL RESIDUE DATA 1999

Rather than restate the levels of the animals in the slaughter classes for 1999, which are essentially similar numbers of animals, it is more important to look at the results of the monitoring program. During this year the monitoring program screened animals for 10 antibiotics: penicillin, streptomycin, tetracycline, tylosin, erythromycin, neomycin, oxytetracycline, clortetracycline, gentamycin, and lincomycin. The target organ for the antibiotic analyses was the kidney. Ten sulfonamides were screened for: sulfamerazine, sulfaquinoxaline, S-bromomethazine, sulfamethiozole, sulfonamide, sulfapyridine, sulfadiazine, sulfadioxime, sulfamethazine, and sulfamethoxazole. The target tissue for these analyses was the liver (Domestic Residue Data Book, 1999).

Table 18.5 displays the results of the monitoring samples in the various slaughter classes and subdivisions in those classes for violative residues. The data in the table show the number of samples taken of the various animals, the number of violations in the sample of animals assayed, the percent of violations, and the statistical upper limit of potential residues as a function of the sampling of animals.

The percentage of violative samples found in horses was 10.3%; there were 46 antibiotic violative (AV) samples from the 446 horses sampled. McGee et al. (2001) indicated that 9% of all horses slaughtered were from feed lots; the remainder were taken from previous ownership and auction. The high incidence of the AV residues found was probably the result of insufficient withdrawal times by previous owners before the animals were brought to slaughter (Grandin et al., 1999). Horsemeat is rarely if at all used for food in the United States.

The combined slaughter classes, which again may be beyond what the FSIS monitoring program intended, indicate that there is a low frequency of AV residues in the samples assayed. The results for the combined categories are: for cattle, out of 1948 samples, there were 2 AV samples, or a 0.10% incidence; in veal calves, a category that one would expect to have a higher violative frequency, out of 1133 samples 17 were AV, a 1.50% frequency. In 558 samples taken for sheep, both mature and lambs, there were no AV samples; in goats, 315 samples were assayed and one or 0.32% was AV. Among swine, another grouping that could have a greater frequency of AV samples, there were only 3 AV samples out of 971 samples assayed or an AV incidence of 0.31%; in chickens, both young and mature, 701 samples were assayed with no AV samples found. Similarly in turkeys, both young and mature, 501 samples were taken and one sample was AV, or a frequency of 0.17%; in ducks no AV samples were found in the 327 samples analyzed.

For the sulfonamide screening samples, the results are as follows: in cattle, 1639 samples were assayed with 2 samples being violative, a 0.12% frequency; in veal calves 1040 samples were assayed with 5 samples violative, an incidence of 0.48%; in sheep (mature), one violative sample was found in 308 samples, or 0.32%; in goats there were no violative samples found in 235 samples. In swine, there were 4 violative samples in 1210 samples assayed, a frequency of 0.33%. In poultry, as a group, there were few sulfonamide violative samples found. In chickens, both young and mature, one sample of 635 was violative, or 0.16%; in 540 turkey assays taken from young and mature birds, there were 2 violative samples, or 0.37%; in ducks there were no sulfonamide violative samples among the 327 samples assayed.

As was noted in the antibiotic and sulfonamide monitoring samples in both the 1998 and 1999 surveys, there was a consistently low frequency of violative residues found across all slaughter groups and across all combined species groupings. There is generally an actual violative rate of less than 1%, the exception being the calf grouping. The statistical upper limits for most of the groupings were below 2%, the exceptions being the calf group and the boars/stags. The boars/stags combination was less than 2.5%, and the calves were below 5%.

TABLE 18.5
Summary of the Monitoring 1999 Sampling of Animals and the Incidences of Violative Frequencies of Antibiotic and Sulfonamide Drugs

Animals	Antibiotics				Sulfonamides			
	Samples	Actual	Percentage	Upper 95% Confidence Limit	Samples	Actual	Percentage	Upper 95% Confidence Limit
Horse	446	46	10.30	13.00	285	1	0.40	1.60
Cattle								
Bull	276	0	0.00	1.40	275	1	0.40	1.70
Steer	459	0	0.00	0.70	466	0	0.00	0.60
Beef cow	292	0	0.00	1.00	291	0	0.00	1.00
Heifer	461	0	0.00	0.60	304	0	0.00	1.00
Dairy cow	460	2	0.40	1.40	303	1	0.30	1.60
Calves								
Bob veal	398	7	1.80	3.30	397	3	0.80	1.90
Fancy veal	316	4	1.30	2.90	316	2	0.60	2.00
Regular veal	191	4	2.10	4.70	151	0	0.00	2.00
Sheep	246	0	0.00	1.20	—	—	—	— ^a
Lamb	312	0	0.00	1.00	308	1	0.30	1.50
Goats	305	1	0.30	1.50	235	0	0.00	1.30
Swine								
Market hog	496	1	0.20	0.90	481	1	0.20	1.00
Boar/stag	207	1	0.50	2.30	323	1	0.30	1.50
Sow	268	1	0.40	1.80	406	2	0.50	1.50
Chickens								
Young	408	0	0.00	0.70	410	2	0.50	1.10
Mature	293	0	0.00	1.00	225	0	0.00	1.30
Turkeys								
Young	411	1	0.20	1.10	412	2	0.50	1.50
Mature	170	0	0.00	1.70	128	0	0.00	2.30
Ducks	327	0	0.00	0.90	249	0	0.00	1.20

^a No data available.

TABLE 18.6
1999 Frequency of Nonviolative Residues of Antibiotics and Sulfonamides in Monitoring Samples

Species	Antibiotics			Sulfonamides		
	Samples	Actual	Percentage	Samples	Actual	Percentage
Horses	446	0	0.00	285	0	0.00
Cattle						
Bulls	276	0	0.00	275	0	0.00
Dairy cows	460	2	0.43	303	0	0.00
Heifers	461	0	0.00	304	0	0.00
Steers	459	1	0.22	466	0	0.00
Calves						
Bob	398	15	3.76	397	1	0.25
Formula-fed	316	51	16.14	316	0	0.00
Nonformula-fed	191	9	4.71	151	0	0.00
Heavy	228	2	0.88	176	1	0.57
Sheep						
Mature	246	0	0.00	— ^a	—	—
Lambs	312	3	0.96	308	0	0.00
Goats	305	0	0.00	235	0	0.00
Chickens						
Young	408	5	1.23	410	0	0.00
Mature	293	3	1.02	225	0	0.00
Turkeys						
Young	411	8	1.95	412	1	0.24
Mature	170	2	1.18	128	1	0.78
Ducks	327	3	0.92	249	0	0.00
Rabbits	204	90	44.18	—	—	—

^a No data available.

The confirmed NV residue picture pointed to the same veal calve “hot spot.” Table 18.6 shows the distribution of NV residues among the slaughter classes. Because none of the residues exceeded the legal tolerance levels, there is no need for the upper 95% confidence frequency levels to be calculated. These data indicate that the animals monitored in a normal sampling of healthy animals at slaughter are quite free of antibiotic and sulfonamide residues. Even in what were called “hot spots,” namely the areas where the incidence is considerably higher than the 1% or less that appears to be a normal frequency, the incidence is relatively low. For example, when the slaughter categories were combined into a more species-related listing, the results only indicate what was seen in Table 18.6. There were but 3 nonviolative antibiotic (NVA) residues samples found in the cattle category in 1656 samples, a frequency of 0.18%; there were no sulfonamide residues found in any of 1348 samples analyzed. In the calves category, there were 77 positive NVA residues found in 1133 samples analyzed, a frequency of 6.67%; only 2 samples of sulfonamide residues were found in 1040 samples, a frequency of 0.19%. In sheep there were 3 positive NVA residues in 558 samples, an incidence of 0.53%; no sulfonamide residues were found in 308 samples analyzed. In goats, of the 305 samples assayed for antibiotic residue, no positives were found; in the 235 samples assayed for sulfonamides, no residues were found. Combining chickens, turkeys, and ducks into a poultry category, 1609 samples were assayed with 21 positive NVA samples found, an incidence of 1.30%; there were only 2 sulfonamide residues found in 1424 samples assayed, a frequency of 0.14%. The real “hot spot” was noted in rabbits where 90 NVA samples were found in the 204 animals sampled,

an incidence of 44.12%. There were no data available on sulfonamide residues. These data reinforce the conclusion that relatively few NV residues are being found in the animals sampled. When this is combined with the violative residue data, it is clear that residues of antibiotics and sulfonamide drugs are becoming less frequent than those found in surveys (Katz and Brady, 1993, 2000) taken 12 to 14 years earlier. This is probably the result of continuous FSIS inspection activity and increased educational efforts related to the proper use of antibiotics in animal agriculture. Continued success of the monitoring and surveillance program of the FSIS resulting in low incidences of both violative and NV residues can only be maintained with the due diligence of regulatory programs. Unfortunately, little reliance can be placed on either voluntary surveillance or quality control.

There are no residue data available for the year 2000. At this writing, the National Residue Program 2000 "Blue Book," which lists the program plans but not the results, remains the latest information readily available.

RESIDUES IN MILK

On December 29, 1989, Ingersoll (1989) reported in the *Wall Street Journal* the results of a survey of milk obtained from 10 cities nationwide for antibiotic and sulfonamide residues. The results used a validated screening assay system and found that some 36% of the milk samples had antibiotic and sulfonamide residues. Only a small percentage contained residues that were above the established tolerance levels. In early 1990, February 7, the CBS-affiliated TV station in New York reported the results of their sponsored survey of 50 samples of milk purchased at supermarkets in the metropolitan NY area covering New York City, the adjoining Connecticut area, and northern New Jersey (Ingersoll, 1990a). Of the samples, 80% contained tetracycline residues and 26% contained residues of sulfonamides. Again, there was only a small incidence of samples containing violative residue levels. In early 1990, the FDA conducted its own national milk survey and reported some 50% of the 70 milk samples taken in 14 cities across the nation contained residues. Shortly afterward, the FDA reported that no antibiotic residues were present in the samples taken in the survey. The *Wall Street Journal* 2 months later reported that the FDA/CVM found 80% of the milk samples actually contained drugs (Ingersoll, 1990b). The Government Accounting Office (GAO) disputed the FDA findings (GAO/RCED-91-26), saying that the FDA could not substantiate claims about milk. The difference in the reporting of the results apparently came from the fact that the screening procedures were far more sensitive than the confirmatory assays. Later in 1990, the FDA/CVM planned a nationwide survey to test milk for antibiotic and drug residues (Ingersoll, 1990c). These surveys pointed up the definition difference between the incidence of NVA and/or antimicrobial residues and violative residues, a problem that does not appear to be totally resolved at this point in time. In the reporting of the data that will follow, there is no definitive information as to the exact levels of the residues.

From these rather sensational media exchanges came the realization that a national system of reporting the results of the milk drug screening programs was necessary. These public media incidents were part of the driving forces resulting in the survey results reported today. Hence, unlike the long-time FSIS program for meat and poultry residues, the comprehensive accumulation of the frequency data for antibiotic and/or antimicrobial residues in milk is a relatively recent occurrence. The national milk residue database is a voluntary, industrially based reporting program. Mandatory reporting is processed through state regulatory agencies via the National Conference of Interstate Milk Shippers (NCIMS), a voluntary organization controlled by member states dedicated to providing a high-quality milk supply to the public and industry. The FDA and the NCIMS collaboratively developed a cooperative federal-state program to compile the results of residue testing and the frequency of residue detection. The database design was developed in consultation with an advisory group consisting of members from the FDA, NCIMS, USDA, dairy producers, dairy processors, state agencies, and academia. The database includes the results of the National Drug

Residue Milk Monitoring program (NDRMMP) and the FDA national milk sampling program. The data are not totally reflective of 100% of the milk from every state.

In addition, the pasteurized milk ordinance (PMO) requires that all bulk milk tankers be both sampled and analyzed for drugs and β -lactam residues before the milk is processed; tankers found to contain drug and β -lactam residues exceeding tolerances (positive milk samples) are rejected for processing into products for human consumption. This is both a good concept and a difficult concept to enforce totally. There are many different families of drugs that can be, or are, used in dairy animals, legally or otherwise. The question as to how many different drugs and/or drug families that can be analyzed, practically, to determine the acceptability of the milk at the processors remains a question to be answered. Milk tank trucks lined up at a dairy must be processed with a reasonable degree of speed and accuracy.

There are four sources of samples: (1) the bulk milk pickup tanker, which contains raw milk from the farm; (2) pasteurized fluid milk and milk products, which includes milk, cream, condensed milk, dry milk products, and condensed and dry whey products; (3) producer samples, which are those taken from a dairy farm; and (4) other sources, which include milk from silos and over-the-road tankers. A sample is deemed positive after being analyzed by a test system acceptable for regulatory action in (1) a certified laboratory by a certified analyst or (2) an initial assay by the milk processor. It appears that a positive is considered a sample that is above the MRL, or safe level. The frequency of the sampling reflects different levels of what can be considered of regulatory importance. Bulk milk samples are required to be taken daily from every tanker; for pasteurized fluid milk and milk products, a minimum of four samples must be assayed for each product, at each plant, every 6 months; for producer samples, each producer must be tested at least four times every 6 months; for the other category, sampling is done on a random basis with no set number of samples taken. Apparently, the concept is to preclude residue-containing milk from entering the dairy; it is felt that the finished product should be relatively free of residues and hence can be sampled relatively infrequently. Similarly, producers should be segregating animals treated for mastitis, ensuring that withdrawal times are being followed and checking milk from treated animals for residues. The producer samples are really the first line of defense and should be assayed more often, not leaving the dairy or processor with the first-line responsibility for excluding residue-containing milk.

The results of two year-long surveys, October 1997 through September 1998 and October 1999 through September 2000, follow. These data give a fair view of the status of the frequency of only violative residues in the U.S. milk supply. Unfortunately, the whole residue picture is not available because the frequency of residues, both violative and nonviolative, is not reported. Regardless, the data speak to increased surveillance and a much improved residue picture in milk. Table 18.7 presents a summary of the results for the combined industrial and regulatory sampling of the 154 billion pounds of milk produced in the 1997 to 1998 period (National Milk Drug Residue Data Base, October 1, 1998–September 30, 1999, October 1, 1999–September 30, 2000).

These data reflect the improvement from earlier experiences wherein surveys of milk sampled (Ingersoll, 1989, 1990a, 1990b) indicated that some 1% of the milk had some sort of violative level residue present. The fact that the incidence of violative residues has dropped at least 10-fold is a remarkable achievement in itself. However, one should not rest on these laurels because there is no information on the overall incidence of antibiotic and/or antimicrobial and/or other drug residues in milk.

The majority of the assays performed were for the β -lactam drugs; 4,344,487 assays of which 4807 were positive, a violative incidence of 0.11%. The aminoglycosides, including gentamicin, accounted for 7176 samples of which only 2 were positive, an incidence of 0.028%. There were 170,329 assays for the sulfonamide drugs with 42 exceeding the tolerance level, an incidence of 0.025%. There were 100,710 assays for the tetracycline drugs with 79 violative samples found, an incidence of 0.078%. The tetracycline assay results are harder to interpret because in the middle of the sampling year, the total tolerance level for the tetracyclines was raised from 80 ppb to 300 ppb.

TABLE 18.7
Results of Industry and Regulatory Sampling, October 1, 1998–September 30, 1999

Source	Industrial			Regulatory			Total		
	Samples	Positives	Percentage	Samples	Positives	Percentage	Samples	Positives	Percentage
Bulk ^a	3,021,763	2573	0.085	20,586	51	0.250	3,042,349	2625	0.086
Past. ^a	5150	0	0.000	54,677	3	0.005	59,827	3	0.005
Prod. ^a	627,747	1079	0.170	188,074	241	0.130	815,821	1320	0.162
Other ^a	88,581	33	0.040	7483	25	0.330	96,064	58	0.060
Bulk ^b	341,075	340	0.100	235	1	0.430	341,310	341	0.100
Past. ^b	404	0	0.000	674	0	0.000	1078	0	0.000
Prod. ^b	76,243	140	0.180	5413	8	0.150	81,656	148	0.181
Other ^b	18,273	17	0.090	1020	0	0.000	19,293	17	0.088
Totals	4,179,236	4182	0.100	278,162	329	0.190	4,457,398	4571	0.101

Note: When tankers pick up milk from both Grade A and non-Grade A producers on the same truck, the milk loads are delivered to a non-Grade A processing facility. These milk loads are reported as non-Grade A. Bulk, tanker; Past., pasteurized; Prod., producer.

^a Grade A milk.

^b Non-Grade A milk.

This increase in the tolerance level could have reduced the number of violative samples. There were no violative residues found in the 556 tests for chloramphenicol, the 1215 tests for the macrolides (erythromycin, tylosin), the 488 for novobiocin, and 11 for pirlimycin. There were a total of 4,624,972 tests performed, of which a total of 4972 were violative positive, an incidence of 0.11%. Of the total number of tests performed, those for the β -lactams accounted for 93.99%; for the sulfonamides, 3.68%; and for the tetracyclines, 2.18%. These 3 families accounted for 99.8% of the assays for antibiotic/antimicrobial residues. The amount of milk sampled represented 0.0722% of the milk produced in that sampling period.

Table 18.8 summarizes the results of the next yearly sampling period and reflects a very similar picture. Single-year successes of a program are not terribly indicative of what one can expect on a continuing basis; the fact that similar results occurred in the following sampling year is very encouraging.

It is interesting to note the breakdown of antibiotic families monitored in this sample year. As both expected and known, the β -lactam drugs were monitored to the greatest extent. Of the 4,565,328 samples assayed, 4,286,169 or 93.9% were for the β -lactam family; 4053 were positives, or 0.09%. Aminoglycosides, which include the streptomycins, neomycin, and gentamicin, accounted for 7980 samples, with 12 positives or 0.15%. Of the 146,414 samples assayed for the sulfonamides, 85 or 0.058% were positive. Although 115,464 samples were analyzed for the tetracyclines and 100 were positive, or 0.086%, this incidence level leaves a significant question mark. In 1998 the tolerance or MRL for the tetracyclines was raised to 300 ppb, three times the European Union (EU) MRL. Prior to that change, 80 ppb was the maximum concentration for combined tetracycline residues; the EU had a combined MRL of 100 ppb. At that time residues were occurring in milk off the supermarket shelves, depending on the weather during the sampling period, with an incidence of 15% to 80% (Kleiner, D.K, Faini, J., and Szember, M., 1988–2000, unpublished results, Rutgers University). The majority of samples approached or were above the tolerance level. Violative samples containing the tetracyclines are essentially nonexistent currently as a result of this change. Hence, the significance of the reported incidence for tetracycline residues gives one a level of assurance considerably less than what it should be. It should not be forgotten that only violative samples are reported in these surveys; it is not the presence alone of the tetracyclines or in a sense the other antibiotics that is reported; only violative samples will keep milk from being accepted for processing. The assays for chloramphenicol (7024), the macrolides (1699), novobiocin (530), and spectinomycin (48) were all negative. The low incidence of violative samples is a good step forward. However, the total incidence of residues would yield data with which one might use to assess the impact of residues and perhaps help understand the link (if any) between residues and the development of antibiotic/antimicrobial resistance in bacteria.

The samples taken during the 1999 to 2000 sampling period represents 0.061% of the total milk supply of 162 billion pounds produced. One can argue whether this program is sufficient to ensure the lowest possible frequency of residues in the milk supply. However, the industry–government–academia approach is perhaps the best practical system to ensure the safety of the milk supply. No system can ensure absoluteness. There is no doubt that samples of milk in violation of existing tolerance levels are at a very low level, at fractions of a percent. Assuming that only the 0.008% of the pasteurized milk that contains violative residues, as measured by the regulatory assays of the Grade A milk, reaches the consumer, a considerable volume is still involved considering there are 162 billion pounds of milk produced, regardless of the fluid milk poundage going into processed milk products and cheeses.

RESIDUES IN EGGS

Unfortunately, no regulatory agency has given eggs the attention this commodity deserves. Many veterinary drugs are used in poultry for both meat birds and egg-producing hens. One of the major barriers to any monitoring program is the lack of standardized and/or validated methodology. With

TABLE 18.8
Results of Industry and Regulatory Sampling between October 1, 1999 and September 30, 2000. Validated and Nonvalidated Assays

Source	Industrial			Regulatory			Total		
	Samples	Positives	Percentage	Samples	Positives	Percentage	Samples	Positives	Percentage
Bulk ^a	3,246,118	2558	0.078	20,344	64	0.310	3,266,462	2622	0.080
Past. ^a	4708	0	0.000	50,401	4	0.008	55,109	4	0.007
Prod. ^a	562,176	973	0.170	143,707	190	0.130	705,883	1163	0.160
Other ^a	90,983	34	0.040	8900	16	0.180	99,883	50	0.050
Bulk ^b	356,515	290	0.080	360	0	0.000	356,875	290	0.080
Past. ^b	25	0	0.000	525	0	0.000	550	0	0.000
Prod. ^b	61,981	108	0.170	1306	0	0.000	63,287	108	0.180
Other ^b	15,951	13	0.080	1328	0	0.000	17,279	13	0.075
Totals	4,338,457	3976	0.009	226,871	274	0.120	4,565,328	4250	0.090

Note: When tankers pick up milk from both Grade A and non-Grade A producers on the same truck, the milk loads are delivered to a non-Grade A processing facility. These milk loads are reported as non-Grade A. Bulk, tanker; Past., pasteurized; Prod., producer.

^a Grade A milk.

^b Non-Grade A milk.

no analytical systems in place, *per se*, and no monitoring system established, there is no national database for antibiotic and/or antimicrobial residues in eggs. Recently, the Canadian Food Inspection Agency (CFIA) (Quon, 2000) monitored both domestically produced and imported (U.S. produced) eggs for veterinary drug residues. The study screened eggs for the presence of chloramphenicol, β -lactams, fluoroquinolones, macrolides, tetracyclines, decoquinatate, holofugizone, and coccidiostats. This screening study surveyed 3569 samples and found 33 (potentially) positive samples, or 0.92%. Eighteen of the 33 positives, 55%, were in eggs produced in the United States. There were no major differences found between sources, Canadian or United States. The residue-containing eggs from the United States contained tetracyclines from eggs from Vermont, Michigan, and Minnesota; sulfonamides from eggs from Maine and Maryland; macrolides and nitromide in eggs from Maine and Minnesota; and ethopabate and clopidol from Maryland.

Any extrapolation made from this study to the frequency of residues in the eggs marketed in the United States is at best tenuous. With a lack of any definitive residue study of market eggs in the United States, however, the estimated frequency of antibiotic and sulfonamide residues would be less than 1%. Using the Canadian data, which indicated that 5 of the 18 positives were not either antibiotic or sulfonamides, the antibiotic and sulfonamide contribution to the total sample was 0.36%. Without the breakdown of what portion of the total sample was of U.S. origin, it is impossible to determine, even with this small study, the frequency of U.S. eggs that had residues.

In the United Kingdom, the Veterinary Medicine Directorate (VMD) claims that 99.3% of poultry meat and 97% of eggs are free of detectable residues. The Soil Association, a group dedicated to organic foods, claims that the VMD provides misleading information concerning the incidence of such residues. The Soil Association claims that a "detailed analysis of the data on which their summaries are based suggests the actual levels could be up to 2000% higher" (Young and Craig, 2002). Rather than impose this discussion into the middle of such a dispute, the logical approach would be to suggest that a publication of all residue data from all nations be prepared for analysis by scientists. However, such a compendium of data will require a great deal of international cooperation and will take considerable time to organize.

The data from the Canadian report are probably somewhat reflective of egg residues in the United States. The conclusion of these authors remains, namely, that something less than 1% of the eggs produced in the United States have measurable residues, the remaining 99% being measurable antibiotic and sulfonamide free. Unfortunately, this conclusion is based on minimal data and should be so interpreted.

RESIDUES IN FISH

There is no national program to determine the extent, if any, of the antibiotic and antimicrobial residue levels found in fish and other seafood commodities marketed in the United States. Information as to what to expect can be gleaned from a listing of feeding studies. With the increasing amount of seafood consumption in the nation, it is logical for a HACCP-based program, similar to that used by the FSIS, to be established for seafood. Needless to say, legislation and assignment of regulatory responsibility are necessary prerequisites.

Withdrawal times from the various species can vary depending on the time of treatment in the growth cycle, the level of treatment, the species, temperature of water, etc. Treatment in many cases is done through the use of medicated feed, which can pose additional ecologic problems such as the sludge-containing drugs generated by fish wastes and uneaten feeds, the development of antibiotic/antimicrobial-resistant bacteria, and the slow release into the aquatic ecosystem of the antibiotic and/or antimicrobial drug from feed not consumed. From a food point of view, the MRL can be set, but there can be significant differences in residue results. For example, the Codex Alimentarius Committee established an MRL for oxytetracycline of 0.10mg/kg. This level seems to be adequate for the black tiger shrimp, *Penaeus monodon*, with a withdrawal time of 14 days

but was not adequate for the freshwater prawn, *Macrobrachium rosenbergii*, which required 21 days (Brillantes et al., 2001). This example only illustrates the general problem; specifics for other aquatic species can also be a variable, hence the need for a systemized regulatory system to ensure that the product does not contain both illegal and potentially harmful residues.

ANTIBIOTICS AND ANTIMICROBIALS IN THE AQUATIC ENVIRONMENT

It is truly remarkable that this problem has never been given its proper placement among the important residue and ecologic problems that beset our environment. The eventual disposition of most drugs used in human medicine and, to a great extent, veterinary drugs will be via excretion. The excreted materials will eventually find their way into the aquatic ecosystem. There is very little knowledge as to the fate of these drugs in the aquatic environment, the ability of the sewage and water purification processes to degrade the medicinals, and the ability of the low levels of drugs to select for antibiotic and antimicrobial resistance. In 1976 the first concerns about drugs in the aquatic environment were voiced when traces of pharmaceuticals were found in the effluent of the Kansas City sewage treatment plant (Hignite and Azarnoff, 1977). Although no drugs were found in the drinking water, the recommendation was made to consider the evaluation of public potable waters; this recommendation was not pursued.

Although Richardson and Bowron (1985) published their findings of pharmaceuticals at measurable concentrations in the aquatic environment in 1985, not until the Daughton and Ternes (1999), Hirsch et al. (1999), Stumpf et al. (1999), Ternes (1998), Herberer and Stan (1997), Herberer et al. (1998), Halling-Sorensen et al. (1998), and Jorgensen and Halling-Sorensen (2000) publications has the research area blossomed and received the interest that it should engender. These reports focus on the chemical families and the concentrations found and allude to the effects on bacteria. There has been little research emphasis placed on the potentials of residue levels of large numbers of drugs on the development of antibiotic and antimicrobial resistance. Concurrent with these published data are numbers of reports in the popular media excerpting meetings where this problem has and is being discussed actively.

The U.S. Geological Survey (USGS) targeted a large number of compounds including the veterinary and human origin antibiotics, the tetracyclines, the fluoroquinolones, the macrolides, sulfonamides, other antibiotics, and coccidiostats. In addition, there are many prescription and nonprescription compounds, insecticides, plasticizers, detergents and related compounds, fire retardants, polycyclic aromatics, antioxidants, fragrances and personal care products, and steroidal hormones present in the waters. The USGS reported a survey (Kolpin et al., 2002) to provide a national assessment of the occurrence of these drugs in streams. The total biological significance of this chemical "soup" remains to be assessed. These drugs and biologically active compounds coupled with the personal care products that are in extensive use define a whole new area of the unknown related to antibiotic resistance development in bacteria.

EFFECTS OF COMBINATIONS OF COMPOUNDS AT RESIDUE LEVELS ON THE SELECTION FOR ANTIBIOTIC RESISTANCE IN BACTERIA

The question as to what is the significance of the multitude of compounds found at low or residue levels on the development of antibiotic resistance in bacteria remains somewhat unanswered and conjectural. A second question to this conundrum is related to the methodologies that will yield some insights into the answer. In a superb discussion of the problems of measuring the effects of antibiotic perturbations of the human intestinal populations, Cerniglia and Kotarski (1999) outlined the advantages and disadvantages of methodologies that have been suggested and used to some

extent (Boisseau, 1993; Corpet, 1987, 1992, 1993; Rollins et al., 1975). Obviously, human testing or animal testing, as long as the animals have intestinal bacterial populations similar to that of humans, would give a greater insight as to what occurs from the ingestion of antibiotics or antimicrobial drugs. The ethical questions of human testing, long experimentation times, large numbers of human subjects, costs, and the lack of ability to study combinations and permutations of drugs makes human testing not at all feasible. Conventional laboratory animals do not have intestinal bacterial populations that resemble those of humans, and extrapolation of results to humans would be difficult. The use of human-associated animals, animals with colonized human bacterial populations, is difficult, requires a gnotobiotic facility, is very expensive, and does not lend itself well to the study of drug mixture interactions. The use of pure cultures, continuous culture systems, and anaerobic cultures of intestinal bacteria does not represent the complex populations of the intestine.

With all the impossibilities mentioned in mind, Brady et al. (1993) and Bordas et al. (1997) used the concept of indicator organisms and the clinical microbiology workhorse, the minimum inhibitory concentration (MIC) methodology to measure the sensitivities or resistances of microorganisms. Indicator organisms have been used in water and food analysis as surrogates for enteric pathogen pollution and contamination; bacteria, yeasts, and cell cultures have been used as indicators of mutagenic potential of compounds. The MIC analytical system measures the sensitivities of microorganisms to specific antibiotic or antimicrobial markers and thus reflects any changes related to antibiotic/antimicrobial and or other drug or compound exposures. Neither type of system was ever intended to simulate or mimic the microbial populations of the intestinal tract. The only question this pure culture system answers is whether exposure of the bacterial culture to single or combinations of compounds changes its antibacterial sensitivities.

Brady et al. (1993) and Bordas et al. (1997) used *Staphylococcus aureus*, ATCC 9144, as the indicator organism and showed that low levels of antibiotics and pesticides, as individual compounds or in combination, were capable of increasing the MIC to several antibiotic/antimicrobial markers. Pesticides, alone and in combination with antibiotics, also raised the frequency of increases in the MIC of the antibiotic markers in an additive manner. The levels of all compounds were 10 ppb or less and hence had relevance to the reality levels found in the environment.

With the increasing awareness of the presence of large numbers of bioactive chemicals in the aquatic environment, Kleiner, (2002) hypothesized that low levels, 10 ppb or less, of antibacterial drugs, pesticides, and veterinary drugs, individually and/or in combination, were capable of increasing the MIC (bacterial resistance) in the indicator bacterium, *S. aureus*, ATCC 9144. He used 17 compounds commonly used in human and veterinary medicine and in agriculture in single, double, and sextuplet combinations. With single compounds, 16.5% of the MIC values were increased by 4 to 8 times, whereas 4.7% were increased greater than 8 times. Increases greater than 4 times were considered substantive. Two-compound groupings increased the MIC values 4 to 8 times 34.3%; 60.6% of the MIC values were increased greater than 8 times. For the sextuplet combinations 15.6% of the MIC marker values increased 4 to 8 times, whereas a remarkable 78.5% of the marker MIC values were increased greater than 8 times. Kleiner concluded that his hypothesis was essentially correct, and therefore bacteria exposed to multiple chemical exposures at low levels (10 ppb) would probably result in the development of or increases in bacterial resistance. These results are not indicative of perturbations in intestinal bacterial populations or a model system for the assessment of antimicrobial, veterinary, and pesticidal compounds on human intestinal bacterial populations. These results only indicate that combinations of chemicals, at residue levels, are capable of increasing bacterial resistance in an indicator organism.

ANTIBIOTIC AND ANTIMICROBIAL RESISTANCE SELECTION CAUSED BY NUTRACEUTICALS

Nutraceuticals have been described by Adeleja (1997) as “naturally occurring or enhanced foods produced and purchased with the belief that the product will promote health or confer a medicinal benefit, including short- or long-term prevention or treatment of disease whether or not the anticipated benefit is claimed or substantiated.” In the United States alone, some 60 million people use herbal products with an estimated market value of some \$80 billion. There have been reports in the media concerning interactions between nutraceuticals and prescription drugs (Fischmann, 2000). There is also a dearth of information related to the development of antibiotic or antimicrobial resistance and how or if nutraceuticals play a role in the inhibition of the microorganisms of the gastrointestinal tract. Unfortunately, the demonstration of safety of the nutraceuticals is not required prior to marketing and any proof of safety or lack thereof is placed on the government. Many nutraceuticals carry with them, although not necessarily on the label, suggestions or claims of possessing antimicrobial activity. Invariably, bacterial exposure to antibiotic or antimicrobial activity usually will result in the development of bacterial resistance to the substances.

Using this concept, Ward et al. (2002) used the aforementioned techniques reported by Brady et al. (1993), Bordas et al. (1997), and Kleiner (2002) with the exception of using the Gram-positive *S. aureus*, ATCC 29213, and the Gram-negative *Escherichia coli*, ATCC 25922. Although these organisms did not possess the same exquisite broad sensitivity to a wide spectrum of antibiotic/antimicrobial drugs as did *S. aureus*, ATCC 9144, both indicator organisms exhibited similar resistance/sensitivity profiles. They established a baseline of the inhibitory activities of the nutraceuticals studied using the MIC as well as the agar diffusion methodologies. These investigations found, in the nutraceuticals studied (aloe vera, Echinacea, garlic, goldenseal, St. John's wort, and zinc), there was a general and analytically significant increase in the MIC of the ampicillin marker in both the *S. aureus*, ATCC 29213, and *E. coli*, ATCC 25922, indicator organisms. The mechanism of these observations remains unclear at this writing. The authors suggested that the purported antibacterial properties of those nutraceuticals may be transitory, have limited effectiveness, and have no effect in the long term.

OVERVIEW

There has been considerable improvement in the residue picture in animals and birds produced for foods. The incidence of violative residues, depending on the species or “slaughter class,” is at least 10-fold less than those found 12 to 14 years previous. Similarly, the incidence of violative residues in milk is down approximately the same factor of 10. Unfortunately, in milk the total residue picture, the percentage of milk containing residues, is not available. In both eggs and seafood, there is at best very limited information. It would be a better situation if the programs for meats and milk were extended to oversee the residue picture in eggs and seafood. The quality of waters and the potentially serious influences of large numbers of chemicals in these waters should be evaluated to ensure the quality of the potable water supply. Both antibiotic resistance and endocrine disruption or influences can be serious environmental as well as public health problems. Nutraceuticals should be studied more thoroughly for their gross and subtle effects, with antibiotic resistance being only one of the potential problem areas.

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19 Update on Hurdle Technology Approaches to Food Preservation

Lothar Leistner and Grahame W. Gould

CONTENTS

Current Applications	621
Amplification of Microbe-Inactivation Techniques.....	623
New Logically Based Hurdle Preservation Processes	624
Prerequisites	626
Predictive Modeling for Hurdle Technologies	626
Microstructure	627
Pitfalls	627
Conclusions	628
References	630

Hurdle technology, the use of combined preservation factors for the gentle, but effective, preservation of a variety of foods, has gained widespread popularity and acceptance in industrialized as well as developing countries. Although originally the development of successful hurdle technology preserved foods, and processes to produce them, were mostly empirically based, improved understanding is now providing more logically based approaches that promise to generate increasing opportunities in the future.

This chapter gives a brief summary of the principles and successful current applications of hurdle technology. However, it also points out the extent of knowledge that is prerequisite for the intelligent application of the techniques and highlights areas where that knowledge is still incomplete and could promote wider application in the future. Furthermore, potential pitfalls in the use of hurdle technologies, possibly encountered by unsuspecting scientists, technologists, and food processors, are mentioned. Thus, this chapter carries on the discussion about the successful introduction of hurdle technology foods beyond the current state of the art. In addition, it aims to assist especially newcomers to this topical and promising field.

CURRENT APPLICATIONS

The microbial safety and stability as well as the sensory and nutritional quality of most preserved foods are based sometimes on one, but more often on several, preservative factors, for which the name “hurdles” has been coined. More than 60 potential hurdles were identified in a European Union Project (Bøgh-Sørensen, 1994), but there are probably hundreds of specific plant- and animal-derived antimicrobials taken into account as well (Leistner and Gould, 2002).

TABLE 19.1
Major Current Applications of Combined Methods (Hurdle Technologies) for Food Preservation Used in Industrialized and Developing Countries

Industrialized Countries

- Minimally processed foods (higher quality; freshlike, and convenient)
- Safer chilled foods (built in safety hurdles; “invisible technology”)
- Fermented foods (in which sequences of hurdles lead to safety and stability)
- Healthful foods (to ensure the safety of foods low in salt, fat, sugar, preservatives)
- Minimally packaged foods (with environmentally friendly packaging procedures)
- Ultraclean packaging procedures (for low contamination of mildly preserved foods)
- Foods with less potentially undesirable preservatives (e.g., sulfite, nitrite)
- Less energy usage for chilling or freezing (by conversion to ambient-stable foods)
- Raw materials decontaminated by multiple hurdles (meat, fruits, vegetables)
- Improved defenses against pathogens (in foods and *in vivo*)

Developing Countries

- South America (especially used for fruit products, also meats and fish)
- China and Taiwan (improved traditional foods, adaptation of fusion foods)
- India (used for variety of dairy, cereal, fruit, poultry, and fish products)
- Africa (preliminary work with juices, fruits, and carcass meat)

For a long time, hurdles were applied empirically to foods by using traditional preservation methods, such as heating, chilling, freezing, drying, salting, conserving, acidifying, fermenting, removing oxygen, and smoking. However, after the principle underlying most of these methods became known, the preservative factors became more scientifically defined — for example, the intensity of high temperature processing (F-value), low temperature (t-value), water activity (a_w), acidity (pH), redox potential (Eh), preservatives, and competitive flora (less well defined). These values are increasingly applied intelligently in advanced hurdle technology. Not only the microbial safety and stability but also the sensory and nutritive qualities of foods are governed by hurdles, which should be well balanced in an optimum range for each type of food (Leistner, 1994a). The *total quality* of food should be secured by advanced hurdle technology. The application of hurdle technology is progressing worldwide for the effective preservation of a large variety of foods. This is true for industrialized countries and for developing ones (Table 19.1). Hurdle technologies in industrialized countries mostly are used to improve food safety, stability, and quality. Hurdle technologies are proving to be more useful in developing countries for the creation of novel foods, which, despite minimal processing, are ambient stable, and for the modification of traditional intermediate moisture food (IMF) products to improve their taste and nutritional value by conversion to higher moisture foods.

For instance, in industrialized countries hurdle technologies are applied for different aspects of food preservation: (1) to achieve safe but minimal processing of foods (freshlike and convenient); (2) to help ensure chilled food safety (design of built-in hurdles that become active in case of temperature abuse); (3) in fermented foods (with a stability and safety often based on a sequence of hurdles); (4) for healthful foods (balanced hurdles that secure microbial stability even in foods with reduced levels of fats, salt, sugar, preservatives, etc.); (5) to encourage less “overpackaged” foods (the replacement of sophisticated but sometimes wasteful “active” or “smart” packaging by more environmentally friendly packaging); (6) further improvements in ultraclean or aseptic packaging that help to foster the application of very mild but still effective hurdle techniques; (7) the development of foods that demand less potentially undesirable preservatives (e.g., sulfite, nitrite); (8) less energy usage (e.g., for chilling or freezing during storage by the introduction of more

ambient-stable, hurdle-preserved foods); (9) microbial decontamination of raw materials such as carcass meat, fruits, and vegetables by microbe-inactivating hurdles (e.g., steam, hot water, alkaline phosphates, lactic and acetic acids) applied as multiple hurdles, but also sequentially; (10) improved and strengthened defense lines against food poisoning (already effective in many foods and thereafter in the human body).

In developing countries, hurdle technology has proved useful in the creation of novel foods. Particularly impressive progress has been made in South America (especially with various fruit products but also with meats and fish), in China and Taiwan (with the improvement of traditional foods and by the adaptation of fusion foods, or a merger between Western and Asian foods), in India (with a variety of dairy, cereal, fruit, poultry, and fish products), and even in some African countries (with juices, fruits, and carcass meat).

All these recently achieved improvements of traditional and novel foods via hurdle technology have been documented and outlined in recent reviews (Leistner, 1999, 2000a; Leistner and Gould, 2002); therefore, details will not be reported here. However, there are areas of hurdle technology that have still received relatively little attention but which, if pursued, will offer improved possibilities in the future (i.e., with respect to the wider use of hurdles as adjuncts in microbe-inactivating techniques).

AMPLIFICATION OF MICROBE-INACTIVATION TECHNIQUES

Most hurdle techniques aim to use combinations of preservation factors to inhibit the growth of microorganisms in foods rather than to inactivate them. However, the possibility of using combinations of factors to amplify the effects of inactivation treatments is potentially very attractive, in particular if it allows reductions to be made to the severity of the inactivation techniques that are being used. There are a number of well-established examples, of course, including the synergistic effect of heat with low pH that allows mild heating to deliver ambient stability of acid-canned foods. Likewise, the use of nisin to prevent outgrowth from spores of thermophiles in thermally processed vegetable foods destined for sale in countries with high ambient temperatures, which allows the use of a milder heat treatment than would otherwise be necessary (Fowler and Gasson, 1991). Multiple combinations (e.g., of slightly reduced pH, reduced a_w , sometimes with nitrite, and with O₂-free packaging) form the basis of many mildly heated, shelf stable meat products (SSP).

There are probably many more possible hurdle combinations that will allow safe reductions in the intensity of microbe-inactivation techniques. Of particular interest, considering the desires of consumers for more “natural” means of food preservation that are less reliant on “chemical additives,” are naturally occurring antimicrobials, which have been shown to be synergistic with heat. These may be usable to reduce the severity of pasteurization and sterilization processes. For example, S-carvone (from caraway seeds) greatly reduced the heat resistance of *Listeria monocytogenes* (Karatzas et al., 2000), and other plant-derived volatile antimicrobials (carvacrol, cinnamaldehyde, thymol, and decenal) had similar effects. It is interesting that similar synergies have been observed between natural antimicrobials and some of the newly emerging microbe-inactivation techniques, such as ultrahigh hydrostatic pressure (UHP) and electroporation. For example, procedures have been reported that allow reductions in the pressure used to inactivate bacteria in UHP processing. These include the addition of nisin (Pol and Smid, 1999) and pediocin (Kalchayanand et al., 1994) and the natural antimicrobial carvacrol (Karatzas et al., 2001), derived from oregano, to reduce the pressure tolerance of *L. monocytogenes* and the spores of *Bacillus cereus*. High pressure transiently sensitized *Escherichia coli* to nisin and also to lysozyme, although *E. coli* is normally resistant to this enzyme (Hauben et al., 1996). Chitosans increased the effectiveness of high pressure against *E. coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* (Papineau et al., 1991). Monoterpenes enhanced the inactivation of *S. cerevisiae* by high pressure (Adegoke et al., 1997). Electroporated cells of *L. monocytogenes*, *E. coli*, and *Salmonella* Typhimurium

became much more sensitive than untreated cells to nisin and to pediocin (Kalchayanand et al., 1994). Most of these synergies were shown to occur in laboratory media but also, significantly, in foods (e.g., carvacrol against *L. monocytogenes* in skimmed milk) (Karatzas et al., 2001), although effects in foods are often disappointingly less than in laboratory media resulting to some extent from binding of the antimicrobials to some food components such as proteins or partition into fats, etc. (Smid and Gorris, 1999).

Such new combination approaches, particularly of natural materials with the novel microbe-inactivation techniques, are introducing attractive new possibilities for future hurdle technologies.

NEW LOGICALLY BASED HURDLE PRESERVATION PROCESSES

Although many hurdle technology-preserved foods were developed empirically, there are increasing numbers of examples of the deliberate application of the principles to develop radically new processing routes and new products. An excellent example is given by the recent CYTED-D Programme (Science and Technology for Development, Fifth Centennial), which involved researchers in seven South American countries and had the objective of developing new hurdle technology preservation systems for a range of tropical fruits, including peach halves, mango slices and puree, papaya slices, whole and sliced pineapple, banana puree, and chichzapote slices (Alzamora et al., 1993), later extended to include other fruits and to encompass not only microbiological stability and safety but other stability and quality aspects, including enzyme activity, color retention, flavor, texture, and so on (Alzamora et al., 2000).

The fully worked out processes were based generally on the combined use of at least 5 hurdles: mild heat treatment (blanching) to inactivate enzymes and to minimize initial microbial contamination; slight reduction of a_w to within the range of 0.97 to 0.92, depending on the fruit and product type, achieved by the addition of sugar; lowering of the pH by the addition of phosphoric and/or citric acids; incorporation, variously, of sorbic or benzoic acids as antiyeast and antimold agents; and addition of metabisulfite as an antimicrobial but more especially to help retain good color. Any microorganisms surviving the processes tended to become “metabolically exhausted” and unable to grow and to die off during storage by a process of “autosterilization” (Leistner, 2000a). High-quality ambient-stable shelf lives reached at least 3 to 8 months, depending on the product.

Many such successful hurdle techniques act principally by interfering with, or completely preventing, homeostatic reactions in microorganisms. These are the many reactions that have evolved to counter a wide variety of environmental stresses to which microorganisms may be subjected (Storz and Hengge-Aronis, 2000) and many of which may be imposed in preserved foods. Homeostatic reactions act first to keep key physiologic systems operating and relatively unperturbed, even when the environment around the cell is greatly perturbed. Second, if the environmental stress is severe, they react further in ways that increase their resistance to the stress. Unfortunately, many mild preservation techniques deliver stresses that elicit such responses. It is, therefore, important that this is taken into account in the design of new logically based preservation procedures.

Table 19.2 lists the major stresses that are important in food preservation and summarizes the nature of the homeostatic responses that must be overcome if effective preservation is to be achieved. Most of the responses are “active” in that they demand new metabolism and the expenditure of energy by the cell. Some may be regarded as “passive” in that they are already built into the structure of the cell when it is formed, as in the dormant, resistant bacterial endospore. Others concern the maintenance of stable populations, or consortia, of cell communities rather than single cells, or “population homeostasis” (Gould, 1995). All play roles in different types of hurdle technologies.

It has been well established with a variety of raw materials (fruits, vegetables, ready-to-eat dishes, carcass meat, etc.) that the reduction of the initial load of microorganisms by various means definitely fosters food preservation and allows that only few and/or mild preservative

TABLE 19.2
Major Stresses and Homeostatic Reactions to Them that Must Be Overcome in Hurdle-Preserved Foods

Stress	Homeostatic Reaction
Active Homeostasis	
Low levels of nutrients	Nutrient scavenging; oligotrophy; "stationary phase response"; generation of "viable nonculturable forms"
Reduced pH	Extrusion of H ⁺ across the cell membrane; maintenance of cytoplasmic pH; maintenance of transmembrane pH gradient and membrane potential
Presence of weak organic acids (e.g., sorbic, benzoic)	Extrusion of additional H ⁺ and sometimes of the acid
Reduced a _w	Osmoregulation; accumulation of "compatible solutes"; avoidance of water loss; maintenance of membrane turgor
Lowered temperature	"Cold shock" response; changes in membrane lipids to maintain satisfactory fluidity
Raised temperature	"Heat shock" response; change in membrane lipids
Presence of biocides	Phenotypic adaptation; reduction in cell-wall/membrane permeability
Ultraviolet radiation	Excision of thymine dimers and repair of DNA
Ionizing radiation	Repair of single strand breaks in DNA
Passive Homeostasis	
High temperature	Built-in mechanisms that maintain low water content in the protoplast of bacterial spores
High pressure	Uncertain; may include adaptation in vegetative cells and low protoplast water content in spores
Electroporation	Low electrical conductivity of the spore protoplast
Ultrasonication	Structural strength of cell or spore wall
Population Homeostasis	
Competition	Formation of interacting communities; aggregates of cells showing some degree of symbiosis; biofilms

Source: Adapted from Gould, G.W. (2000).

factors (hurdles) are sufficient for microbial stability and safety of the products. Furthermore, the decontamination of the raw materials proved to be more effective if several preservative factors (multiple hurdles) are used to remove as many as possible of the microorganisms initially present, and especially pathogens. For instance, research into carcass decontamination technologies (including steam or hot water vacuuming; spraying/washing/rinsing before evisceration and/or chilling, using water or solutions of chemicals and/or steam or hot water) has demonstrated that combination treatments applied sequentially as "multiple hurdles" are the most efficacious (Sofos et al., 1999). Bacon et al. (2000) evaluated such "multiple-sequential interventions," including steam vacuuming, preevisceration carcass washing, and rinsing with solutions of organic acids. Their results strongly supported the use of sequential decontamination processes in beef packing plants as means for improving the microbiological quality of carcasses. Combinations of 3 or 4 treatments were more effective than single or double treatments in reducing the risk of transmission of pathogens from cattle to consumer.

In the past such decontamination procedures were viewed with some suspicion because they might cover up hygienic shortcomings. However, if high hygienic standards, based on Good Manufacturing Practice (GMP), in addition to effective decontamination procedures are used, the risk of pathogens (e.g., *E. coli* O157:H7, *Salmonella*) entering the food chain could be greatly reduced.

PREREQUISITES

Preservation technologies for foods are based mainly on the inhibition of microbial growth or on microbial inactivation. However, also physical and chemical changes lead to quality deterioration. If a food should be optimally preserved by the application of hurdle technology, knowledge of the usual microbiological, chemical, and physical deteriorations of a food is a prerequisite. Furthermore, preservative factors that are suitable to secure the safety and the quality of the food have to be carefully chosen. This demands knowledge of the nature and implementation of the many hurdles known by now. To apply preservative factors sensibly, with respect to their quality and intensity, it is essential to know their effects and limits to inhibit or inactivate relevant microorganisms and their side effects on the sensory and nutritional quality of the food (Leistner and Gould, 2002). These data are well established for the major preservative factors (e.g., high and low temperature, reduced a_w and pH, several preservative additives). A further prerequisite for successful food preservation is some knowledge of the basics of hurdle technology. As mentioned earlier, a good understanding of the homeostatic reactions of microorganisms to stresses; the metabolic exhaustion of stressed organisms; and the basis for the multitarget preservation of foods, which should be the ultimate goal, will foster advanced food preservation and avoid pitfalls (Leistner, 2000b). In particular, developments in predictive microbiology should increasingly support the application of soundly based hurdle techniques.

A user's guide for the design of hurdle technology foods was created some time ago (Leistner, 1994b) and has proved suitable for solving real product development tasks in the food industry, but it is clearly open to further improvements. The user's guide is based on a linkage between hurdle technology, predictive microbiology, and the Hazard Analysis Critical Control Point (HACCP) concept (or, if more appropriate, quantitative GMP guidelines). The user's guide could be further developed by taking account of recent experimental and practical results (i.e., in the predictive microbiology applied not only to kinetic, but also to boundary or probability models, because these are specially relevant to hurdle preserved foods).

PREDICTIVE MODELING FOR HURDLE TECHNOLOGIES

A sound knowledge of the effectiveness of particular combinations is fundamental to the success of hurdle-based preservation. In the past, much of that knowledge was empirically derived. Now, however, advances in predictive microbiology are beginning to offer a more logically based approach.

Predictive microbiology attempts to describe the effects of environmental conditions on the growth, survival, and death of microorganisms. Much food-related predictive microbiology has been concerned with conditions that affect the growth of food poisoning bacteria and has concentrated on kinetic modeling, allowing the prediction of lag times prior to the initiation of growth and generation times during the subsequent logarithmic phase. Kinetic models have been very useful, especially for predicting the safe shelf lives of chill-stored foods and especially because a number of computer software systems are now available for the major pathogens and the effects of the principle preservation factors on them (Buchanan, 1993; McClure et al., 1994). However, kinetic models are less useful close to the boundary between growth and no growth, and less useful therefore for establishing the conditions necessary for long ambient stability (Baranyi and Roberts, 2000). More recently, therefore, increasing attention has been given to the development of models that allow prediction of the probabilities of growth under different conditions and of models that allow prediction of the growth–no growth boundaries or the growth–no growth interface (Ratkowsky and Ross, 1995). More attention is being given to food spoilage microorganisms as well as to food poisoning ones. For example, Presser et al. (1998) used the boundary approach to determine the growth–no growth interface for *E. coli* as a function of temperature, pH, lactic acid, and a_w . Likewise, McKellar and Lu (2001) developed a probability of growth model for *E. coli* O157:H7 as a function

of temperature, pH, NaCl, and acetic acid that correctly predicted growth–no growth for 99% of the points in 1820 treatment combinations studied. Using literature data, the model correctly predicted the growth outcomes in 25 out of 26 sets of conditions. The growth–no growth boundary for *L. monocytogenes* in Mexican-style cheeses was defined for combinations of the hurdles pH, NaCl, and moisture content by Bolton and Frank (1999), and for pH, NaCl, and lactic acid by Tienungoon et al. (2000). Because spoilage yeasts are often the most tolerant microorganisms in low pH, low a_w foods, even if containing a preservative such as sorbic acid, they have been important targets for recent probability and boundary modeling. López-Malo et al. (2000) undertook probability modeling of *S. cerevisiae* as a function of pH, sorbic acid, and a_w . Jenkins et al. (2000) modeled time to growth and probability of growth of the extremely tolerant *Zygosaccharomyces bailii* under conditions generally relevant to acidified foods. López-Malo and Palou (2000) modeled the growth–no growth interface of *Z. bailii* more specifically as a function of the hurdles relevant to the preservation of mango puree.

These probability of growth, and growth–no growth boundary approaches, are complementary in that the growth–no growth interface is defined by the conditions under which the probability of growth becomes so low that it can confidently be assumed to be zero. The recent increase in interest in boundary modeling is to be welcomed because it is highly supportive of the development of many hurdle technology preservation systems, and particularly those that aim to generate safe and stable foods that are intended to have long ambient-stable shelf lives. There are, however, influences on microbial growth and survival in foods that are not covered by the more well-known factors, and these include effects of food structure.

MICROSTRUCTURE

There is one potentially important and exploitable hurdle that remains poorly understood, with one notable exception. This is food microstructure. Controlled microstructure impedes growth of microorganisms in some foods to the extent that it provides the most important hurdle. This is most obviously so in butter, margarines, and low-fat spreads, in which dispersion of the aqueous phase as small droplets within the continuous lipid phase forms the basis of the effective preservation of such products (Verrips, 1989). However, the effects of food microstructure on the potential for growth of microorganisms in other types of products remain poorly understood, although electron microscopical techniques have already indicated that there is much to learn (Wimpenny et al., 1995). Although numeric values for factors such as temperature of storage, intensity of thermal or pressure processing, pH, a_w , and to a lesser extent E_h are widely used, there are no well-accepted numeric values that can be exploited regarding the effects of structure, or microstructure, on microorganisms in foods, with the exception of the water-in-oil emulsions. Numeric values, for instance, that relate meaningfully to gel strength, viscosity, diffusivity, tortuosity, effects of rubbery/glassy states, or other materials science parameters, and their influence on microbial growth, would probably add yet another range of hurdles for intelligent manipulation and exploitation. This must represent an attractive area for future collaborative research between microbiologists, histologists, physicists, and materials scientists.

PITFALLS

Hurdle technology is not an easy remedy to make foods safe and stable, but it provides a powerful framework to improve the microbiological and quality aspects of many foods. Traditional foods, such as intermediate moisture foods with a_w s below 0.80, or canned foods thermally processed according to the minimum 12D concept, are often overprocessed, resulting in safe products but with inferior quality. Advanced hurdle technology foods should not be overprocessed or underprocessed. Their hurdles should be tightly controlled so as to be “right on target.” This means that the preservation factors used must be well understood and intelligently applied. For instance, chemical

preservatives should not be added in excessive amounts, which would lead to “chemical overloading” of the food. This has particularly been a problem in some developing countries. However, it is desirable to apply several hurdles, aiming at several “target classes” at the same time, because of the likely benefit of synergistic rather than simple additive effects. The use of several hurdles in combination does not necessarily mean the addition of several chemical preservatives because this could create labeling problems. Rather, attempts should be made to combine chemical hurdles with physical ones (e.g., heating, chilling, drying, fermenting, vacuum or modified atmosphere packaging, etc.), which do not require labeling.

It is well established that microorganisms can adapt to hostile environments, and by genetically controlling stress reactions, they may increase their resistance and, if pathogenic, their virulence. Furthermore, enhanced resistance against one hurdle can result in “cross resistance” against others. It has been suspected that such cross resistances are important potential pitfalls in the application of sublethal hurdles. However, most such adaptations have been investigated for only one hurdle at a time. Stress reactions could be better overcome, or avoided, if several hurdles are applied at the same time because then the homeostatic activities within the microbial cells become more difficult and more energy demanding if several targets (e.g., membrane activities, osmotic and pH control, specific enzymes, protein synthesis, DNA replication, etc.) are disturbed at the same time. Ultimately, metabolic exhaustion will occur, and the cells will die (Leistner, 2000b).

In general, novel or modified hurdle technology foods should be carefully designed and their microbial safety and stability must be thoroughly tested using “challenge tests” combined with relevant predictive modeling, especially if these foods should be ambient stable.

CONCLUSIONS

Hurdle technology, applied for the mild and effective preservation of foods, has proved to be successful in numerous instances, and many of the decisive principles of this topical method of food preservation have already been elucidated. P.M. Davidson (personal communication, 2002) has pointed out that hurdle technology is a good example of a holistic approach to food preservation. Progress should be made in deciding and then quantitatively predicting hurdle effects, and more should be learned about the mechanisms of action of individual and combined hurdles.

Often, when multiple hurdles are used, they act at different targets within the cell, exemplifying the attractive principle of “multitargeting” (Leistner, 1995a). This principle has long been fundamental to the successful development of antimicrobial drugs and other products within the pharmaceutical industries (Denyer and Hugo, 1991) but has been less clearly recognized in food preservation microbiology. However, many of the examples of successful hurdle technology preservation (see Table 19.1) result from this approach, even if not realized at the time that the foods and processes were developed.

The underlying rationale for the multitarget approach is now more easily understood and can often be put on a sound scientific basis (Leistner, 2000b). The sort of analysis summarized in Table 19.2 can provide a logical framework for the identification of logical multitargets and help to guide the development of new and improved hurdle techniques. A simple illustrative example is given in Table 19.3 for the preservation of a hypothetical foodstuff. Each hurdle is applied to the extent and by means that are optimally compatible with the particular food (i.e., with respect to taste, texture, appearance, etc.). First, any reduction in pH, with acids chosen for optimum compatibility (e.g., phosphoric, citric, malic, acetic, etc.), will begin to elicit an energy-requiring homeostatic response in the target microbial cells because they must export any excess of H^+ that leaks into the cell down the concentration gradient that has been established across the cell membrane. Next, addition of a lipophilic weak organic acid will accelerate leakage of H^+ into the cell, as well as interfere with membrane functions, so that an even greater energy demand is made. Next, reduction of a_w by the addition of optimally compatible solutes (mixtures of salts, sugars,

TABLE 19.3
Logical Application of Hurdles

Applied Hurdle	Major Effect
Reduce pH	Forces cell to expend energy expelling H ⁺ that leaks into the cell
Add lipophilic organic acid	Further increases the leakage of H ⁺ into the cell and also interferes with membrane function
Reduce a _w	Forces cell to osmoregulate by synthesizing or accumulating compatible solutes
Reduce O ₂ ; vacuum or modified atmosphere packaging	Prevent the growth of strict aerobes; reduce the energy available to facultative anaerobes for the necessarily enhanced export of H ⁺ and osmoregulation that the hurdles have imposed
Consider additional product-compatible stresses (e.g., heat, cold, pressure, bacteriocins and culture products, and other naturally-occurring antimicrobials)	
Take account of autosterilization, the continued reduction in numbers of microorganisms during storage of some types of hurdle-preserved, ambient-stable foods	
Consider exploiting autosterilization if necessary, using quarantine to further ensure safety by encouraging the die-off of any pathogens that may occasionally contaminate a particular food	

polyols, etc.) and/or partial drying, or the incorporation of dry ingredients, will force the microbial cells to osmoregulate, adding further metabolic demands. Then, it is logical to remove O₂, by vacuum or modified atmosphere packaging, because this will reduce the energetic efficiency of facultatively anaerobic microorganisms, so that they are less able to react to the combined pH-, organic acid-, and a_w-stresses that have been imposed on them. And, if O₂ levels are greatly reduced, growth of strict aerobes, including most molds, will be prevented completely. Finally, maybe the logical sequence can be usefully extended further by the intelligent use of additional hurdles (e.g., mild heat or pressure), incorporation of product-compatible natural antimicrobials, and so on. A relevant innovative example from a developing country is the development of ambient-stable paneer in India, which depends on 5 classical hurdles. These are temperature (mild heating to F-value 0.8), reduction of pH to 5.0, reduction of a_w to 0.95, addition of 1000 ppm sorbate, and reduction of Eh (packaging in hermetically sealed containers). The product kept well for 30 days at 30°C (Rao and Patil, 1999). A relevant example from industrialized countries is the traditional processing of fermented sausages (salami) in which 5 hurdles are applied in sequence: addition of nitrite-curing salt to the sausage batter, reduction of E_h, growth of competitive flora (lactic acid bacteria), reduction of pH, and reduction of a_w. This leads to stable and safe products (Leistner, 1995b).

Altogether, such logically based, multitarget approaches should increasingly be used for the development of future mild, but effective, hurdle technology preservation systems.

Application of hurdle technologies in food preservation is a fast-moving and dynamic field, so that further improvements are feasible and likely. For instance, the new and emerging technologies that are currently coming into use, or being explored, mostly aim to allow preservation without the use of extreme heat. These technologies, including high hydrostatic pressure, electroporation, and high-intensity light, initially were considered as “standalone” procedures. However, it has already turned out that maximum benefit will probably be obtained when the techniques are used in combination with more traditional hurdles. In this sense, the hurdle technology approach is highly relevant to such future developments in food preservation. Therefore, it might be concluded that hurdle technologies will remain key to food preservation in the future.

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20 Mechanisms of Action, Resistance, and Stress Adaptation

A.D. Russell

CONTENTS

Mechanisms of Action of Antimicrobial Agents.....	634
Potential Target Sites	634
Effects of Antimicrobials on Outer Cell Layers	635
Effects on the Cytoplasmic (Plasma) Membrane.....	637
Leakage of Intracellular Constituents	637
Gross Membrane Disruption.....	639
Dissipation of Proton-Motive Force	639
Inhibition of Membrane Enzymes	640
Sulfites as Membrane-Active Agents.....	640
Antimicrobials Interacting with Cytoplasmic Constituents	641
Alkylating Agents.....	641
Cross-Linking Agents.....	641
Intercalating Agents.....	641
Interaction with Cytoplasmic Protein	641
Mechanisms of Microbial Resistance to Antimicrobials	642
Definitions of Terms	642
Microbial Cell Impermeability.....	642
Bacterial Spores.....	643
Mycobacteria	644
Gram-Negative Bacteria.....	645
Yeasts and Molds	645
Efflux of Antimicrobial Agents	645
Acquired Resistance Mechanisms	646
Mutational Resistance	646
Plasmid-Mediated Resistance	646
Biofilms and Microbial Response to Antimicrobials.....	647
Cellular Inactivation of Antimicrobials	647
Biocide and Antibiotic Resistance in Bacteria.....	648
Stress Adaptation.....	649
References	651

Antimicrobial agents are chemically diverse. They consist essentially of (1) chemotherapeutic drugs, for example, antibiotics, and (2) biocides, a collective term used increasingly to describe compounds with antiseptic, disinfectant, and/or preservative activity (Russell and Chopra, 1996). In addition

(3) many compounds that possess antimicrobial activity occur naturally in plants and animals (Board, 1995; Nychas, 1995).

This presentation will concentrate on biocides (Hugo and Russell, 1994). Antibiotics used in the chemotherapy of human or animal infections will not be considered. However, there is a possible association between biocide usage in the medical, food, and other areas and antibiotic resistance and due attention will be made to such a linkage (Lambert et al., 2001; Randall et al., 2001).

It is important to know how antimicrobials act on a variety of microorganisms and equally how microbes resist or otherwise overcome the actions of these chemical agents. These aspects form the basis of this chapter.

It is impossible to cover all types of microorganisms here. Instead, the emphasis will be placed on microorganisms that are of special significance in foods or in the food industry, notably Gram-positive bacteria (including spores), Gram-negative bacteria, and yeasts and molds (Gould and Russell, 1991a; Russell and Gould, 1991).

The focus is essentially on antimicrobials added to, or present naturally in, foods. However, antimicrobials may also be used as disinfectants or sanitizers within the food industry, and thus compounds such as quaternary ammonium compounds (QACs), iodophors, and chlorine-releasing agents (CRAs) will also be considered where relevant.

MECHANISMS OF ACTION OF ANTIMICROBIAL AGENTS

There are two possible ways of examining the mechanisms of action of antimicrobial agents. The first is to consider a range of compounds individually. The second approach is to decide the possible targets within microbial cells and discuss how antimicrobials interact with them. The latter provides a better understanding and comparison of the ways whereby different antimicrobials achieve their effect and is thus the system adopted here.

POTENTIAL TARGET SITES

There are several sites (Table 20.1) on or within a cell at which an antimicrobial could act. These depend not only on the types of microorganisms but also on the nature of the chemical agent (Russell, 1984; Denyer and Stewart, 1998).

Thus, in Gram-positive cocci, potential target sites include the cell wall, cytoplasmic membrane, functional and structural proteins, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA). The same potential targets sites occur in Gram-negative bacteria except that the outer membrane replaces

TABLE 20.1
Potential Target Sites on or within Microbial Cells

Type of Microorganism	Potential Target Sites
Cocci	CW, CM, proteins, enzymes, DNA, RNA
Gram-negative bacteria	OM, IM, proteins, enzymes, DNA, RNA
Mycobacteria	CW, CM, proteins, enzymes, DNA, RNA
<i>Bacillus</i> spp. and <i>Clostridium</i> spp.	OSC, ISC, cortex, spore membranes, spore core
Molds	CW, CM, proteins, enzymes, DNA, RNA
Yeasts	CW, CM, proteins, enzymes, DNA, RNA

Note: CW, cell wall; CM, cytoplasmic membrane; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; OM, outer membrane; IM, inner membrane; OSC, outer spore coat; ISC, inner spore coat.

the cell wall. Mycobacteria possess probably the same or similar targets, but the picture here is complicated by the accessibility of these sites to inimical agents (Russell, 1996). It is interesting that bacteriostatic and mycobacteriostatic concentrations are often similar, whereas mycobactericidal concentrations are usually much higher than bactericidal ones (Russell, 2002a).

Bacterial spores are found mainly in the genera *Bacillus* and *Clostridium*, both of which have immense importance in food microbiology. The response to biocides of cells depends on their stage in the overall cell cycle. Mature spores are the least sensitive, whereas, depending on the actual antimicrobial, other forms (outgrowing, germinating, or vegetative cells) are much easier to inactivate. Clearly, changes occur as spores give rise to vegetative cells and as the latter sporulate to account for such differences. At first sight, this implies that target sites disappear as others arise or that target sites change, although mutation is not a cause. For example, the configuration of DNA is different in spores (low water content) from that found in vegetative cells (higher water content). Large amounts of low-molecular-weight basic proteins, small acid-soluble spore proteins (SASPs), are present in the spore core (protoplast) that are rapidly degraded during germination (Setlow, 1994; Tennen et al., 2000; Loshon et al., 2001). These SASPs appear to play an important role in determining spore sensitivity to several antibacterial agents (Setlow, 1994; Setlow et al., 2002). The spore core also contains RNA, DNA, and dipicolinic acid (DPA), as well as most of the calcium, potassium, manganese and phosphorus, whereas the cortex consists largely of peptidoglycan, including a spore-specific muramic lactam. The spore coat(s) may provide target sites at which antibacterial agents act but are more likely to present a barrier to their entry. By contrast, the cortex, core, and the inner and outer forespore membranes may be associated with the mechanisms of action of sporicides.

EFFECTS OF ANTIMICROBIALS ON OUTER CELL LAYERS

A primary interaction occurs at the microbial cell surface that could be, but usually is not, responsible for inhibition or a loss of viability (Table 20.2). The medically important “chemosterilizer” glutaraldehyde (1, 5 pentanedial) interacts strongly with surface groups. More relevant in the context of food antimicrobials, the activity of QACs is strongly dependent on external pH. An increase in pH results in the cell surface becoming more negatively charged so that binding of the positively charged QAC molecules is enhanced. With Gram-negative bacteria, it is believed that sufficient damage to the outer membrane may occur thereby enabling these antimicrobials to promote their own entry into the cell (Russell and Russell, 1995; Russell and Chopra, 1996). In Gram-positive bacteria such as staphylococci, it is believed that QACs are able to enter the cells readily, and no self-promotion is likely to be needed.

TABLE 20.2
Damage by Antimicrobial Agents to the Outer Layers of Microbial Cells

Antimicrobial Agent	Type of Microbial Cell	Outer Layers Involved	Possible or Actual Damage
Ethylenediamine tetraacetic acid (EDTA)	Gram-negative bacteria	OM	Removal of Mg ²⁺ , release of some LPS
Lysozyme	Gram-positive bacteria	Peptidoglycan	β-1, 4-linkages
Glutaraldehyde	Gram-positives, Gram-negatives	Cell wall/OM	Cross linking of proteins
Chlorhexidine salts	Gram-negatives	OM	Displacement of cations? Self-promoted entry
QACs	Gram-negatives	OM	Displacement of cations? Self-promoted entry

Note: OM, outer membrane; LPS, lipopolysaccharide; QACs, quaternary ammonium compounds.

Chelating agents such as ethylenediamine tetraacetic acid (EDTA) have long been known (Russell, 1971) to increase the permeability of Gram-negative bacteria to a range of chemically diverse compounds. Used usually as the disodium salt, EDTA extracts lipopolysaccharide (LPS), protein, and lipid from the outer membrane of susceptible cells as a consequence of its chelation of stabilizing inorganic cations, notably Mg^{2+} (Temple et al., 1992a,b; Ayres et al., 1993, 1998). EDTA-susceptible organisms include *Pseudomonas aeruginosa*, *P. stutzeri*, and *Escherichia coli*, including *E. coli* O157:H7 (Hathcox and Beuchat, 1996). The activity of EDTA is greater at alkaline than at acid pH, and this reflects its enhanced chelating ability in the presence of Tris and lysozyme. However, cell inactivation can be prevented by the incorporation into the system of a nonpenetrating solute such as sucrose. The resulting spheroplasts can be used for various laboratory investigations.

Although originally claimed (Russell, 1971) to have little or no effect on Gram-positive bacteria, subsequent studies have demonstrated an inhibitory effect of EDTA on the growth of *Staphylococcus aureus* and *Enterococcus faecium* (Kraniak and Shelef, 1988; Bobey et al., 2000). These organisms differ from the Gram-negative ones referred to earlier by possessing little cell-envelope (wall) cation. The effects of EDTA thus must be dissimilar in nature, and it is conceivable that EDTA inhibits growth of Gram-positive bacteria by forming stable chelates in culture media with multivalent cations that are essential for bacterial growth (Kraniak and Shelef, 1988).

Other chelating agents related to EDTA have a similar potentiating or inhibitory effect on bacteria (Haque and Russell, 1974a,b). In addition, chemical agents that are believed to act through their chelating properties such as citric acid, gluconic acid, polycations, and sodium hexametaphosphate all affect the permeability of the outer membrane of Gram-negative bacteria (Vaara and Vaara, 1983; Ayres et al., 1993, 1998). More recently, polyethyleneimines have been studied; these are available in different molecular masses and forms and are used to facilitate purification of soluble proteins by flocculating cellular contaminants from cellular homogenates. Polyethyleneimine (PI), a weakly basic aliphatic polymer that is polycationic in nature has potent permeabilizing activity against a range of Gram-negative bacteria (Helander et al., 1997, 1998) and potentiates the activity of hydrophobic antibiotics (Helander et al., 1998) and of various biocides against such organisms.

The antibacterial activity of the enzyme, lysozyme, has been known for many years. It is a narrow-spectrum enzyme, some Gram-positive bacteria being susceptible, whereas Gram-negative organisms such as *P. aeruginosa* and *E. coli* are not susceptible, although they can be sensitized by exposure to chelating agents such as EDTA, as pointed out earlier. Lysozyme targets cell-wall peptidoglycan, hydrolyzing the β -1-4 linkages between the amino sugars, with cell lysis ensuing. This cell disintegration can be prevented by the presence of a relevant concentration of a nonpenetrating solute such as sucrose, a classical method for preparing protoplasts of bacteria such as *Micrococcus lysodeikticus* and the nonsporulating form of *Bacillus megaterium* (McQuillen, 1960). Spheroplasts of Gram-negative bacteria can be produced by a combination of lysozyme, Tris buffer, and EDTA, as pointed out earlier. In the context of food microbiology, lysozyme has another role to play. It has been found that the incorporation of lysozyme into recovery media increases the counts of heat-treated *Clostridium perfringens* types A, B, C, and D spores (Cassier and Sebald, 1969; Labbe and Chang, 1995) and of heated *C. botulinum* spores types B, G, and F (Sebald and Ionesco, 1972; Peck et al., 1992a,b, 1993, 1995; Stringer et al., 1995). The D-values at 90°C of *C. perfringens* are about 2-fold to 2.8-fold greater when the enzyme (1 μ g/ml) is included in the recovery medium (Labbe and Chang, 1995). With *C. botulinum*, a diphasic response has been observed (Peck et al., 1992a; Lund and Peck, 1994) such that 1% to 2% of heat-treated spores respond to lysozyme, thereby demonstrating the presence of lysozyme-permeable and lysozyme-impermeable spores in the population. It has also been found that treatment of heated spores with sodium thioglycollate prior to plating in agar containing lysozyme increases the number of colonies (Peck et al., 1992a; Lund and Peck, 1994). Thioglycollate is responsible for the breakage of disulphide bonds in the spore coats, thereby rendering the spores more permeable to the enzyme, which acts on the cortex peptidoglycan.

TABLE 20.3
Membrane-Active Agents

Membrane-Active Agents	Type of Organism	Mechanism of Action
Phenols	NSB	Generalized membrane damage
Chlorhexidine	NSB	Concentration-dependent effects, membrane integrity affected
QACs	Yeasts	
	NSB	
Sorbic acid	Yeasts	
	NSB	Effect on proton-motive force: transport inhibitor
Parabens	NSB	Transport inhibitor (low concentration), membrane integrity affected (high concentrations)

Note: NSB, nonsporulating bacteria; QACs, quaternary ammonium compounds.

EFFECTS ON THE CYTOPLASMIC (PLASMA) MEMBRANE

The cytoplasmic (plasma) membrane is a delicate, semi-permeable membrane situated beneath the cell wall/outer membrane of bacterial or fungal cells. As such, damage to the membrane (unless repaired) can lead to microbial inactivation (Table 20.3).

The cytoplasmic membrane is composed of lipids and protein (in fungi, sterols may also be present). The membrane is normally perceived as a fluid mosaic model in which globular proteins are embedded in a phospholipid matrix or bilayer. Both the protein and phospholipid components can be targets for antimicrobial attack (Russell and Chopra, 1996).

Several chemically diverse antimicrobial agents produce injury to the cytoplasmic membrane, although the type of damage may vary. They include alcohols, QACs, phenolics, organic acids and esters, and isothiazolones. They are often referred to as “membrane-active agents,” a rather loosely used term because it takes no account of the possible effects of antimicrobials on the outer membrane of Gram-negative bacteria. Perturbation of homeostatic mechanisms can also be achieved by physical processes that include mild heat shock, and greater cellular damage can be brought about by chemicals used at elevated temperatures.

Damage to the cytoplasmic membrane can take several forms, namely leakage, lysis, dissipation of the proton-motive force (PMF), and enzyme inhibition. Each of these will be considered separately.

Leakage of Intracellular Constituents

Damage to the cytoplasmic membrane usually results in the release of intracellular constituents, the first being potassium (K^+) leakage followed by inorganic phosphates (Pi), pool amino acids, and then larger molecular weight material (RNA/DNA) indicative of gross injury. QACs generally induce more rapid leakage from Gram-positive than Gram-negative bacteria because the outer membrane in the latter microorganisms acts as a permeability barrier (Russell and Chopra, 1996; McDonnell and Russell, 1999).

Leakage can best be envisaged as a measure of the generalized loss of function of the membrane as a permeability barrier; it may be related to bacteriostasis or fungistasis but not necessarily to cell inactivation, although higher biocide concentration might be expected to be associated with the latter event. However, a diphasic pattern of leakage may occur in which the extent of release of intracellular constituents is actually reduced at higher concentrations, as with QACs, because of a sealing off of the membrane (Hugo, 1999). QACs combine with membrane phospholipids and thereby perturb homeostasis by bringing about membrane disruption. Anionic surfactants, by

contrast, interact with the protein moiety of the membrane; high concentrations (2%) of sodium lauryl sulphate dissolve the cell envelopes of *E. coli* (Hugo, 1999).

The importance of concentration must be emphasized. Nonantibiotic antimicrobial agents can exert several different effects on microorganisms, and these may depend on the actual concentration of a particular compound. It is thus necessary to delineate cellular changes that may occur at low, intermediate, and high concentrations. This aspect has been particularly well covered by Hugo (1999), who demonstrated that low concentrations might have delicate effects on microbial cells but without necessarily being inhibitory or lethal, although such effects could contribute to the damage inflicted at higher biocide levels. Hugo has also emphasized that inhibition of a specific enzyme is unlikely to be responsible for cell inactivation. This is especially relevant at the present time in light of studies that suggest that the mechanism of action of the bisphenol (phenylether), triclosan, resides in its ability, at low concentrations, to inhibit the enzyme enoyl reductase (McMurry et al., 1998). However, other effects, including membrane damage, are believed to be responsible for bacterial inactivation at higher triclosan concentrations (Suller and Russell, 1999, 2000).

The effects of concentration can be quantified in terms of the concentration exponent (dilution coefficient, η), which provides a measure of the role that concentration or dilution has to play in the activity of a particular biocide (Russell and McDonnell, 2000). Thus, antimicrobials with a high η -value (e.g., phenolics *ca.* 6) will rapidly lose activity on dilution, whereas those with a low η -value (e.g., QACs *ca.* 1) will retain much of their activity on dilution (Russell, 1999a). This theme has been developed by Hugo and Denyer (1987), who here attempted to correlate η -value with the known mechanisms of action of several biocidal agents. This interesting concept is summarized in Table 20.4, from which the following can be seen:

1. Chemical agents with low η -value (Group A) often act as -SH (sulfhydryl) reactors or interact with proteins and nucleic acids or are powerful membrane disrupters.
2. Those with intermediate η -value inhibit transport inhibitions (Group B).
3. Compounds with high η -value also act as membrane disrupters but only at high concentrations (Group C).

TABLE 20.4
Possible Relationship between Concentration Exponents and Postulated Mechanisms of Action of Some Antimicrobial Agents*

Group	Antimicrobial Agent	Concentration Exponent	Postulated Mechanism of Action
A	Hydrogen peroxide	1–2	-SH reactor +
	QACs		Membrane disrupter
	Formaldehyde		Interacts with protein and nucleic acid
	Iodine		-SH reactor
	Chlorine		(oxidation) +
B	Parabens	2–4	- SH reactor (oxidation) +
	Sorbic acid		Transport inhibition
	Benzoic acid		(effect on proton-motive force) +
C	Aliphatic alcohols	>4	Membrane disrupter
	Phenolics		Membrane disrupter

Note: +, Other actions also known.

* Based on Hugo and Denyer (1987).

Several alcohols are rapidly bactericidal even to acid-fast bacteria (Russell, 1996). Ethanol and isopropanol (Seiler and Russell, 1991) disrupt the cytoplasmic membrane, probably as a result of penetration into the hydrocarbon interior. Ethanol has pleiotropic effects on bacteria, and inhibition of macromolecular biosynthetic processes is believed to be a secondary effect following membrane damage (Seiler and Russell, 1991). Other effects that include inhibition of enzymes involved in glycolysis, fatty acid and phospholipid synthesis, and solute uptake result directly from the ethanol-induced disruption of membrane structure and function (Seiler and Russell, 1991). Phenylethanol and phenoxyethanol also induce a generalized loss of membrane function but have other more specific effects also.

Organic acids (e.g., benzoic, sorbic, and esters [*para*-hydroxybenzoates or parabens]) will also induce intracellular leakage (Furr and Russell, 1972a,b). They also possess other effects on microorganisms (Eklund, 1989; Kabara and Eklund, 1991).

Gross Membrane Disruption

Although extensive cytoplasmic membrane damage may be caused, antimicrobial agents do not necessarily produce cell lysis. However, bacterial spheroplasts and protoplasts and yeast protoplasts are lysed by biocides that cause gross membrane damage (Hugo and Longworth, 1964; Hiom et al., 1992).

QACs, phenolics, alcohol, anionic surfactants, and parabens (at high concentrations) are examples of antimicrobial agents that lyse morphologic variants (Russell and Chopra, 1996; Hugo, 1999). The selectivity of membrane action can be demonstrated by studying the effects of biocides on protoplast or spheroplasts suspended in various nonpenetrating solutes. For example, lysis by tetrachlorosalicylanilide and trichlorocarbanilide occurs in ammonium nitrate and ammonium chloride, respectively, because of increased permeability to NO_3^- and Cl^- . By contrast, QACs produce protoplast lysis in various solutes because they have a generalized rather than specific effect on the membrane (see Russell and Chopra, 1996, for a discussion).

Dissipation of Proton-Motive Force

The chemiosmotic theory propounded by the British biochemist Peter Mitchell envisages a mechanism in which active transport, oxidative phosphorylation, and adenosine triphosphate (ATP) synthesis can be explained. These processes are powered by a PMF generated by the electron transport chain and ATP hydrolysis. The PMF is expressed as a gradient across the cytoplasmic membrane, and in bacteria the interior of the cell is alkaline and negatively charged in relation to the external environment (Mitchell, 1961). Mathematically, the chemiosmotic theory can be expressed as follows:

$$\Delta\rho = \Delta\psi - Z\Delta\text{pH}$$

in which $\Delta\rho$ represents the PMF, $\Delta\psi$ represents the membrane electrical potential, ΔpH represents the transmembrane pH gradient, and Z represents a constant that converts pH values to millivolts. $Z (=2.303 \text{ RT/F})$ has a value of 61 at 37°C (Mitchell, 1961).

Several antimicrobial agents are capable of dissipating the PMF (Denyer and Stewart, 1998) including the following:

1. Organic acids and esters used as preservatives. Some organic lipophilic acids, notably benzoic, sorbic, and dehydroacetic acids, are used as food preservatives as are the parabens. Organic acids may act either as a source of carbon for microorganisms or may be inhibitory, depending on (i) the concentration of acid and the pH, (ii) ability of the acid to enter the microbial cell, and (iii) the capacity of a microorganism to metabolize it.

pH is a major factor affecting activity of organic acids, and at pH values greater than the pK value (the pH at which 50% ionization occurs) antimicrobial activity decreases rapidly (Sofos et al., 1986). Although it was at one time believed that the undissociated acid was totally responsible for the antimicrobial effect, it is now realized that the behavior of an acid inside the microbial cell must also be considered. Over an external pH range of 5.5 to 9, the internal pH (pH_i) (i.e., of the cytoplasm) is strictly regulated so that molecules of an organic acid will dissociate almost completely, assuming that dissociation in the cytoplasm is similar to that found in aqueous solution. Thus, it is now considered that both the proton and the anion contribute to the overall effects of an organic acid. Lipophilic acids inhibit the active uptake of amino and oxo acids in *E. coli* and *B. subtilis* (Eklund, 1980). Sorbic acid affects the PMF in *E. coli*, and it appears that it dissipates $\Delta\rho$ across the membrane and inhibits solute transfer but has a smaller effect on the membrane potential ($\Delta\psi$) (Salmond et al., 1984; Eklund, 1985).

The parabens also inhibit active transport and selectively inhibit ΔpH across the membrane. These occur at low paraben concentration, with higher levels affecting membrane integrity (Eklund, 1985).

2. Organic acids used as acidulants. Organic acids used in this context lower artificially the pH of foods and include acetic, citric, and lactic acids (Booth and Kroll, 1989; Brown and Booth, 1991). Microbial growth is usually limited at lower pH, and this can be enhanced by adding lower concentrations of organic acid preservatives. Sorbic and acetic acids have similar pK_a values but the former is much more inhibitory; in addition, organic acids used as preservatives are more potent microbial inhibitors than are other weak acids of similar pH (Booth and Kroll, 1989). The mechanism of action cannot be explained solely by the alteration of pH_i and may cause protein denaturation.
3. Alcohols. Proton translocation in *E. coli* is induced by phenoxyethanol at low concentrations; higher levels produce gross membrane damage (Gilbert et al., 1977).
4. QACs. At concentrations that inhibit the growth of *S. aureus*, cetrimide discharges the pH component of $\Delta\rho$ (Denyer and Hugo, 1977). Benzalkonium chloride inactivated cells of *L. monocytogenes*, inhibiting energy metabolism and producing total depletion of ATP pools (Luppens et al., 2001). Exponential phase cells were more susceptible than stationary phase cells (Luppens et al., 2001, 2002).

Inhibition of Membrane Enzymes

The cytoplasmic membrane is rich in enzymes, such as those associated with the electron transport chain that use the electrochemical potential of protons to power active transport. Hexachlorophane (hexachlorophene) specifically affects the electron transport chain. Several other agents interact with thiol (sulfhydryl, -SH) groups in enzymic and structural protein. Thiol groups derived from cysteine residues are essential for the activity of many enzymes and reaction with, or oxidation of, these groups by antimicrobials results in cell inhibition or inactivation. Thus, metals such as mercury and silver interact directly with -SH groups to produce mercaptides (or equivalent); bronopol oxidizes thiol groups to disulfides; and oxidizing agents, such as halogens, progressively oxidize thiol groups to disulfides, sulfoxides, or disulfoxides. Isothiazolones interact with thiol groups, sometimes with the formation of several different products (Collier et al., 1990).

Sulfites as Membrane-Active Agents

Sodium metabisulfite (mainly), sodium sulfite, and sodium bisulfite are used to generate sulfur dioxide and related anions for use as preservatives in foods and beverages (Gould and Russell, 1991). Sulfites can exist in solution as (1) $SO_2 \cdot H_2O$, which predominates at very low pH (0 to 2); (2) the bisulfite ion, HSO_3^- , which predominates at higher pH (approx. 3 to 6), (3) sulfite ion, SO_3^{2-} .

The mechanisms of the antimicrobial action of sulfites have been widely studied (Gould and Russell, 1991; Rose and Pilkington, 1989). Their activity is greater at low pH values and it is probable that only molecular SO₂ can cross the cytoplasmic membrane. In yeast, this is believed to occur via passive rather than active transport, and it is possible that they interact with the lipid and especially the protein moiety of the membrane.

ANTIMICROBIALS INTERACTING WITH CYTOPLASMIC CONSTITUENTS

Several antimicrobials (e.g., QACs, chlorhexidine) produce noticeable interaction with cytoplasmic constituents only at high concentrations (Hugo, 1999). Others alkylate proteins and/or nucleic acids, and still others produce cross-linking effects (Russell and Chopra, 1996).

It is thus often difficult to pinpoint an exact target site within a cell at which such agents act; more likely, such antimicrobials have multiple sites, the effects on which will contribute to the eventual cell inactivation.

Alkylating Agents

Biological activity of alkylating agents is indicated by reaction with nucleophilic groups. Alkylation is defined as the conversion of H-X into R-X, in which R is an alkylating group. Vapor-phase disinfecting and sterilizing agents such as ethylene oxide, propylene oxide, and β -propiolactone all act as alkylating agents (Michael and Stumbo, 1970). In addition, formaldehyde as a gaseous or liquid antimicrobial acts in a similar manner, although it also possesses cross-linking properties (Gorman et al., 1980). Ethylene oxide, for example, combines with the amino, carboxyl, sulfhydryl, and hydroxyl groups in bacterial proteins and also interacts with the N-7 guanine moieties of DNA (McDonnell and Russell, 1999).

Cross-Linking Agents

Formaldehyde is an extremely reactive chemical and combines readily and strongly with protein, RNA, and DNA; interaction with DNA is responsible for its mutagenic activity (Gorman et al., 1980). Intermolecular cross-links are produced when formaldehyde interacts with protein or nucleic acids, but the monoaldehyde also acts as an alkylating agent in its interaction with the amino, carboxyl, sulfhydryl, and hydroxyl groups found in proteins (reviewed by Hugo, 1999; McDonnell and Russell, 1999). Another potent cross-linking agent is glutaraldehyde, its interaction with proteins involving the α -amino groups of amino acids; this is pH-dependent (Gorman et al., 1980).

Intercalating Agents

Antibacterial dyes comprise the acridines and the triphenylmethane groups of compounds such as crystal violet. The latter are much more active against Gram-positive than Gram-negative bacteria. Acridines (e.g., proflavine, aminacrine) are weakly active against both types of organisms, but the dyes in total are not sporicidal. Triphenylmethane dyes and acridines are more active at alkaline pH (Foster and Russell, 1971; Hugo and Russell, 1999). The triphenylmethane compounds interact with acid groups, in particular nucleic acids, within the cell; however, intercalation is not involved.

The acridines, by contrast, combine with several sites in the bacterial cell, the most important of which is DNA. This occurs via intercalation of an acridine molecule between two layers of base pairs (Foster and Russell, 1971; Wainwright, 2001).

Interaction with Cytoplasmic Protein

High concentrations of several antimicrobials (e.g., QACs, phenolics, chlorhexidine) cause cytoplasmic protein coagulation (Hugo, 1999; Russell and Chopra, 1999). Iodine compounds, including

TABLE 20.5
Mechanisms of Microbial Resistance to Antimicrobial Agents

Resistance Mechanism	Type of Organism	Antimicrobial Agent(s)
Impermeability ^a	Bacterial spores	CHX, QACs, Phenolics
	Mycobacteria	CHX, QACs
	Gram-negative bacteria	QACs
	Yeasts and molds	CHX?
Efflux	Gram-negative bacteria	CHX, QACs, triclosan
	Staphylococci	CHX, QACs
Mutation	Gram-negative bacteria	CHX, QACs, triclosan
	Staphylococci	CHX, QACs
Phenotypic adaptation to intrinsic resistance (biofilms)	Several types of microorganisms	Range of antimicrobials
Biocide degradation	Bacteria, fungi	Possible: triclosan, phenolics, CHX, QACs

Note: CHX, chlorhexidine salts; QACs, quaternary ammonium compounds.

^a See also Table 20.6.

the iodophors, combine with thiol groups in proteins but also react with bacterial cytoplasmic protein (Russell, 1999). Iodine is far more active at acid than at alkaline pH, the active moiety being diatomic iodine (I₂) with some contribution from hypoiodous acid (HI).

MECHANISMS OF MICROBIAL RESISTANCE TO ANTIMICROBIALS

Attention is increasingly being devoted to improving our understanding of the ways in which microorganisms evade the action of antimicrobials (Stickler and King, 1999). Mechanisms of bacterial and fungal resistance (Table 20.5) have been extensively researched but are examined here only insofar as a consideration of a possible association with insusceptibility to food preservatives and disinfectants.

DEFINITIONS OF TERMS

There is often disagreement about the terminology applied to microbial insusceptibility to antimicrobials. By analogy with antibiotics, a strain is said to be resistant if it is not inactivated (or, in some instances, inhibited) by a concentration of biocide that inhibits the majority of strains of that organism; a strain may also be considered resistant if not inactivated (possibly inhibited) by a concentration of biocide that is used in practice, the in-use concentration. In addition, the cells in a culture are stated to be resistant if they are not inactivated (possibly inhibited) by a concentration that inactivates (inhibits) the majority of cells in that culture. The terms “tolerant,” “tolerance,” “insusceptibility,” and “reduced susceptibility” are also used by some authors. An innate form of resistance, intrinsic resistance, might in fact be more usefully referred to as intrinsic insusceptibility (Russell, 2001b).

Microbial Cell Impermeability

Uptake of an antimicrobial into a microbial cell is usually necessary for that agent to exert an inhibitory or lethal effect. The word “usually” is used deliberately because some antimicrobials

TABLE 20.6
Microbial Cell Impermeability as a Mechanism of Resistance to Antimicrobials

Type of Microorganism	Mechanism of Intrinsic Resistance	Examples of Antimicrobials
Bacterial spores ^a	Spore coats and possibly cortex	CHX, QACs, phenolics
Mycobacteria	Waxy cell wall	CHX, QACs
Gram-positive cocci	Unknown	Generally, much more sensitive than Gram-negatives
Gram-negative bacteria	OM as barrier	QACs
Yeasts	Cell wall	CHX? QACs?

Note: CHX, chlorhexidine salts; QACs, quaternary ammonium compounds; OM, outer membrane.

^a See Table 20.8 for more detail about spore resistance mechanisms.

have a powerful effect at the cell surface that could be mainly responsible for or contribute to their effects on microorganisms. Consequently, the nature of the cell surface as a possible barrier that limits the uptake of an antimicrobial must be evaluated (Table 20.6; see also Ishikawa et al., 2002). As pointed out earlier, the cell surface differs considerably in various types of microorganisms and thus the reason for their relative responses will be dissimilar. Too little attention has previously been devoted to this issue (Russell et al., 1997).

Bacterial Spores

Bacterial spores of the genera *Bacillus* and *Clostridium* are invariably the most resistant of all types of bacteria to antimicrobial agents (Russell, 1982, 1990; Bloomfield, 1999). Many antimicrobial are bacteriostatic (sometimes bactericidal) and sporostatic at low concentrations while not being sporicidal even at high levels, whereas others are sporicidal at elevated concentrations albeit requiring long periods of contact (Table 20.7). The former category is exemplified by alcohols, QACs, and phenolics and the latter by formaldehyde, glutaraldehyde, and CRAs (Russell, 1990, 2001a). The complex structure of bacterial spores, in particular the nature of the spore coats, has a role to play in reducing the uptake of several antimicrobial agents, although it must be added that little is known about the manner in which such compounds enter the spore. The spore coats appear to have a definitive role in limiting uptake, although the cortex also plays an important part (Table 20.8).

Studies by Setlow and coworkers (Tennen et al., 2000; Loshon et al., 2001) have demonstrated that α,β -type SASPs can coat the DNA in wild-type spores of *B. subtilis* thereby protecting it from attack by enzymes and antimicrobial agents. Spores ($\alpha\beta^-$) that lack these SASPs are much more sensitive to hydrogen peroxide and hypochlorite (Setlow, 1994).

Loshon et al (2001) have also discussed the importance in resistance of the reduced permeability of the spore core to hydrophilic agents, the reduced water content in the core, and the repair of DNA damage during germination (Williams and Russell, 1992a,b, 1993a,b,c). Because different antimicrobials have different mechanisms of action, the importance of the different resistance factors varies with the agent in question (Loshon et al., 2001).

Germinating and outgrowing spores (Table 20.7) tend to be considerably more susceptible to antimicrobial agents than mature spores (Bloomfield and Arthur, 1994; Bloomfield, 1999). There are many cellular changes that take place during these processes that account for this altered response, notably the degradative changes in the outer spore layers during germination such that the developing cell approaches the vegetative state.

Permeability to antimicrobials probably increases and certainly the uptake of these compounds is enhanced when compared to mature spores. The cells are thus inactivated more rapidly than

TABLE 20.7
Possible and Actual Sporicidal and Sporistatic Mechanisms of Action of Antimicrobials^a

Mechanism of Action	Example(s)
Sporistatic	
Germination trigger mechanism inhibited	Sorbic acid
Spore germination inhibited	Phenolics, parabens
Outgrowth inhibited	QACs, chlorhexidine
Sporicidal	
DNA damage	Formaldehyde, alkylating agents
Spore germination abolished	Iodine-based disinfectants
Spore inner membrane modified to become nonfunctional in germinates spore	Oxidative agents
One or more key but unidentified spore proteins	Peroxides

Note: QACs, quaternary ammonium compounds.

^a Based, in part, on Russell (1990), McDonnell and Russell (1999), and Loshon et al. (2001).

TABLE 20.8
Mechanisms of Bacterial Spore Resistance to Antimicrobials^a

Spore Component/Property	Mechanism of Resistance
Coat (s)	Impermeability
Cortex	Impermeability?
Core	Reduced permeability to hydrophilic agent
Core	Reduced water content
α,β -type SASPs	Protection of spore DNA
DNA damage	Repair during germination

Note: SASPs, small acid-soluble spore proteins.

^a Based on Russell (1990), McDonnell and Russell (1999) and Loshon et al. (2001).

spores. What is still unknown is why germination is insusceptible to some inhibitors (e.g., chlorhexidine, QACs) while being inhibited by others (e.g., phenolics, parabens, organic acids) (Russell, 1990).

Mycobacteria

Mycobacteria are generally less resistant than bacterial spores but more so than other nonsporulating bacteria to antiseptics, disinfectants, and preservatives (Russell, 1996). The most likely reason for this response is the complex nature of the mycobacterial cell walls that provide an effective barrier to the entry of such compounds (Table 20.6). The mycobacterial cell wall consists of a mycoylarabinogalactan–peptidoglycan skeleton (see Russell, 1996, for a review). Cell-wall composition may be influenced by the environmental niche of a particular species (Barry and Mdluli, 1996).

Antimicrobial agents that have powerful activity include phenolics, peracetic acid, hydrogen peroxide, and alcohol (McDonnell and Russell, 1999), whereas others, including QACs, are mycobacteriostatic even when used at high concentrations (Russell, 1996). However, new formulations

of QACs are claimed to be mycobactericidal. Generally speaking, current evidence points to the mycobacterial cell wall, especially the arabinogalactan component, acting as a permeability barrier to the entry of biocides (Russell, 1996; McDonnell and Russell, 1999). Other components, such as mycolic acids, have been implicated in cell-wall impermeability presented to some antibiotics, and it is conceivable that future studies will provide data to show a similar role against biocide uptake.

Gram-Negative Bacteria

Gram-negative bacteria are generally less susceptible to biocides than are nonsporing, nonmycobacterial Gram-positive bacteria (Russell and Gould, 1988). There is a marked difference in the susceptibility of *S. aureus* and *E. coli* to QACs, hexachlorophene, and diamidines, but little difference in sensitivity to chlorhexidine. However, *P. aeruginosa* and *Burkholderia cepacia* are considerably more resistant to most of these agents, and *Proteus* and *Providencia* species possess an above-average resistance to cationic biocides such as QACs and chlorhexidine (Russell and Gould, 1988). The outer membrane of *P. aeruginosa* is responsible for its high resistance, with the high Mg^{2+} content helping to produce strong LPS–LPS links and the small size of porins limiting general diffusion. The high content of phosphate-linked arabinose in the LPS of *B. cepacia* decreases the affinity of its outer membrane for cationic and polycationic antibiotics and biocides (Cox and Wilkinson, 1991). The presence of a less acidic type of outer membrane LPS in the cells of *Proteus* species could account for the resistance presented to chlorhexidine and QACs (Vaara, 1992). *Providencia stuartii* strains with low intermediate and high resistance to chlorhexidine have been studied but no gross differences in the chemical composition of the outer cell layer have been found and the inner membrane does not appear to be implicated (Ismael et al., 1986).

Impermeability (Table 20.6) is thus a major intrinsic insusceptibility mechanism in Gram-negative bacteria. However, another intrinsic mechanism that has been insufficiently studied is efflux, and multidrug efflux pumps have been shown to be associated with the insusceptibility of *E. coli* to various biocidal agents (Sulavik et al., 2001). The interplay between impermeability reducing uptake and the efflux of low intracellular concentrations needs to be explored further.

Yeasts and Molds

The amount of information about the manner in which fungi circumvent the actions of biocides is sparse (Dekker, 1987; Lyr, 1987; Russell and Furr, 1996; Russell, 1999a). Intrinsic resistance (Table 20.6) may be associated with the barrier presented by the fungal cell wall, although the available evidence to support this contention is limited. The porosity of the yeast (*Saccharomyces cerevisiae*) cell wall is affected by its chemical porosity, the wall then acting as a barrier or modulator to the entry and exit of various chemicals (De Nobel et al., 1989, 1990). Studies with chlorhexidine (Hiom et al., 1992, 1995a,b, 1996) showed that the relative porosity of cells decreased with aging with a reduced uptake of radio-labeled chlorhexidine gluconate; there was also a significant increase in cell-wall thickness. It was concluded, on the basis of these and other findings, that glucan and mannoproteins play a key role in determining the uptake and activity of chlorhexidine. *C. albicans* is less sensitive and takes up less ^{14}C -chlorhexidine gluconate (Hiom et al., 1995a). Similar studies need to be conducted with other biocides and a range of yeasts and molds.

Other possible intrinsic resistance mechanisms include enzymatic biocide inactivation, efflux, and phenotypic modulation (Rose, 1987; Warth, 1988; Russell and Furr, 1996; McDonnell and Russell, 1999).

EFFLUX OF ANTIMICROBIAL AGENTS

Efflux is an increasingly important mechanism of bacterial resistance to biocides. Effectively, this is the removal of an intracellular level of a biocide that would prove toxic to the cell. However, as

will become clearer later, the efflux of an antimicrobial agent has to be set against the actual concentration of an antimicrobial. While lower concentrations of a compound may thus be pumped out of a cell (and this could account for the elevated minimum inhibitory concentrations (MICs) found with certain strains), at higher concentrations a competition would ensue between damage inflicted on a cell by a biocide producing inactivation and an efflux pump desperately trying to remove the toxic element. At such levels, inactivation of the cell takes place rather than removal of biocide. Efflux may be associated with both intrinsic and acquired resistance mechanisms (Levy, 1992, 2002; Poole, 2001, 2002).

ACQUIRED RESISTANCE MECHANISMS

Mutational Resistance

A major reason for bacterial resistance to antibiotics is associated with mutational changes in cellular target sites. Thus, the target site is insensitive to the inhibitor but is still able to perform its normal physiologic function. Examples are provided by bacterial resistance to β -lactams, aminoglycosides, quinolones, tetracyclines, and glycopeptides (Russell and Chopra, 1996).

Fewer studies have been made to determine whether chromosomal gene mutations confer resistance to antiseptics, disinfectants, and preservatives. Some 50 years ago, it was shown (Chaplin, 1951, 1952) that *Serratia marcescens* normally inhibited by a QAC at $<100 \mu\text{g/ml}$ in broth could adapt to grow at 100 mg/ml , although exactly how such a high concentration could be incorporated into the medium was not made clear. Resistant and sensitive cells, the former possessing an increased lipid content, showed different surface properties. The resistance was unstable, however, being lost when the resistant cells were grown in QAC-free media. Since then, there have been several reports of the acquired resistance of bacteria to biocides, although this insusceptibility may or may not be stable (Gilbert and McBain, 2001; McBain and Gilbert, 2001; Russell, 2001b).

Stepwise exposure of *P. aeruginosa* to the polypeptide antibiotic polymyxin results in cells resistant to this drug and to EDTA (Gilleland et al., 1974). The cells have a defective self-promoted uptake pathway and contain increased amounts of H1, a major outer membrane protein, but a corresponding decrease in envelope Mg^{2+} . Because identical phenotypes appear in cells growing in Mg^{2+} -deficient media, it has been proposed (Gilleland et al., 1974) that H1 replaces Mg^{2+} at cross-bridging sites with LPS. These sites are normally those at which interaction occurs (EDTA) with Mg^{2+} at which displacement of Mg^{2+} (polymyxin) takes place.

Cells of *E. coli* resistant to alcohol have been isolated in which the increased insusceptibility is the result of a decrease in the ratio of inner membrane phosphatidylethanolamine to the anionic phospholipids, phosphatidylglycerol and phosphatidylidglycerol (see Seiler and Russell, 1991).

Plasmid-Mediated Resistance

There have been numerous reports that link the presence of plasmids in bacteria with increased tolerance to chlorhexidine, QACs, triclosan, diamidines, and acridines (Chopra, 1991; Russell, 1997). Some antiseptics and disinfectants are, on an MIC basis, less active against strains of *S. aureus* containing a plasmid encoding resistance to the antibiotic gentamicin. The role of QAC genes in conferring low-level resistance to cationic biocides is considered later.

Based on DNA homology, it has been proposed (Rouche et al., 1990) that *qacA* and related genes carrying resistance determinants evolved from preexisting genes responsible for normal cell transport system. It has further been suggested that the antiseptic resistance genes evolved prior to the introduction and use of cationic biocides (Littlejohn et al., 1990)

Plasmid-encoded resistance to inorganic and organic mercury compounds has been known for many years (Foster, 1983), although this is of much greater relevance in the clinical context because

organomercurials may still be used as pharmaceutical preservatives. Plasmid-encoded resistance to silver has been found in various Gram-negative bacteria (Trevor, 1987). Bacterial resistance to formaldehyde and other industrial biocides may be plasmid-encoded (Candal and Eagon, 1984). Mechanisms of formaldehyde resistance are either (1) alteration in outer membrane proteins (Kaulfers et al., 1987) or (2) formaldehyde dehydrogenase activity (Kaulfer and Masquardt, 1991; Kummerle et al., 1996).

Plasmid-mediated efflux pumps provide an important mechanism of resistance to many antibiotics, metals, and cationic antiseptics and disinfectants (Paulsen and Skurray, 1993; Seiler and Russell, 1991).

BIOFILMS AND MICROBIAL RESPONSE TO ANTIMICROBIALS

Sessile bacteria on surfaces or present within biofilms are less susceptible to biocides than are planktonic cells (Costerton et al., 1987, 1994; Lewis, 2001). It has been known for several years that nutrient limitation and reduced growth rates can modify the susceptibility of bacteria to antimicrobials (Brown and Williams, 1985), and these factors are important parameters in determining the response of sessile organisms.

Biofilms can be considered (Costerton et al., 1987) as consortia of bacteria and possibly other microorganisms contained in an extensive mucoexopolysaccharide polymer or glycocalyx. Biofilms are produced as a consequence of an association of microorganisms with solid surfaces, the sessile organisms differing phenotypically from planktonic cells (Brown et al., 1995; Morton et al., 1998).

Bacteria in biofilms are more resistant than planktonic cultures to several different types of biocides, including iodine and iodophors, CRAs, glutaraldehyde, and chlorhexidine (Morton et al., 1998). However, concentrations of hydrogen peroxide well below those required for total disinfection can remove biofilms (Christensen et al., 1990).

The reason for the greater resistance of bacteria within biofilms remains to be fully elucidated. Nevertheless, several possibilities can be considered: (1) reduced access of antimicrobial agents to the cells within a biofilm, (2) chemical interaction between the biofilm and the molecules of the biocide, (3) modulation of the microenvironment, (4) production of degradative enzymes, (5) genetic exchange between cells, and (6) quorum sensing. Another possibility has been described, namely that persister cells in biofilms are responsible for the intransigence of biofilm cells to biocides (Spoering and Lewis, 2001). Hypochlorite transport into biofilms is retarded, but bacteria within a biofilm are considered to be protected by some mechanism other than physical shielding by the biofilm matrix (Stewart et al., 2001).

Biofilms thus provide an important example of how physiologic (phenotypic) adaptation can play a role in conferring intrinsic insusceptibility to biocides and antibiotics. However, organisms removed from a biofilm and recultured in culture medium are generally no more insusceptible than ordinary planktonic cells.

CELLULAR INACTIVATION OF ANTIMICROBIALS

The enzymatic inactivation of antibiotics such as β -lactams is a well-known phenomenon and an important resistance mechanism. By contrast, microbial inactivation of biocidal-type agents is unlikely to be a major mechanism that confers resistance. Most such antimicrobials at in-use concentrations tend to be recalcitrant to enzymatic breakdown, although much lower concentration may be more susceptible (Beveridge, 1999).

It has been claimed, however, that some microorganisms can degrade triclosan in the environment (Meade et al., 2001). Confirmation of this finding in relation to triclosan insusceptibility awaits further investigation.

BIOCIDE AND ANTIBIOTIC RESISTANCE IN BACTERIA

Biocide and antibiotic resistance in bacteria might be linked (Russell, 1999b, 2000, 2001b). Although this is undoubtedly of concern in the clinical (Russell, 1999b) and possibly domestic (Gilbert and McBain, 2001) context, it also merits serious consideration from those areas in food microbiology in which preservatives or disinfectants are used.

Several aspects need to be discussed. For instance: (1) are antibiotic-resistant bacteria more resistant to biocides, (2) are biocide-resistant bacteria more resistant to antibiotics, and (3) do biocides at in-use concentrations or biocide residues select for drug-resistant organisms? These and other questions have been posed previously (Russell, 2000, 2001b). There is little evidence of antibiotic-resistant bacteria being less susceptible to in-use biocide concentrations (Rutala et al., 1997) or the converse (Russell, 2000). What must be considered, therefore, above all else, is the possible relationship between biocide usage and biocide and antibiotic resistance and, especially in the present context, how it relates to food microbiology.

Chemical disinfectants have a useful role to play in preventing or controlling cross-contamination and infection in the domestic environment, particularly when used in food hygiene and in a healthcare situation (Bloomfield and Scott, 1997). Some antimicrobials such as triclosan may be included in a variety of products, triclosan itself being incorporated into food chopping boards as well as several healthcare products. Specific preservatives may also be permitted in some foodstuffs. The possibility arises, therefore, as with the clinical situation (Russell, 2002a,b,c) that disinfectants and preservatives could present a selective impetus toward the development and isolation of antibiotic-resistant bacteria (White and McDermott, 2001).

The QACs are widely used as disinfectants in medical and food environments (Sundheim et al., 1998). As pointed out by Heir et al. (1998), the prevalence of biocide-resistant food-related microorganisms is of concern to the food industry. QAC resistance in clinical and food strains of staphylococci is encoded by various *qac* resistance genes, viz. *qacA*, *qacB*, *smr* (formerly *qac/qacD*), *qacG*, and *qacH* (Heir et al., 1998). The corresponding gene products, respectively *QacA*, *QacB*, *Smr*, *QacG*, and *QacH* proteins, are responsible for efflux of low concentrations of lipophilic cationic compounds. It has been suggested (Heir et al., 1998) that the spread and maintenance of these genes in staphylococci isolated from clinical and food environments (i.e., the food industry) has occurred because of the selective pressure brought about by their widespread usage. Increased resistance to β -lactam antibiotics of a methicillin-resistant *S. aureus* (MRSA) strain as a result of mutations conferring resistance to the QAC benzalkonium chloride has been described (Akimitsu et al., 1999). Oddly, although the MIC for the parent MRSA was 5 mg/L and 10 mg/L for a series of benzalkonium chloride-resistant mutants, MICs of several β -lactam increased by up to 500-fold. The MIC of benzalkonium chloride against a methicillin-sensitive *S. aureus* (methicillin-susceptible *S. aureus*, or MSSA) strain was 0.7 mg/L. The QAC-resistant MRSA mutant showed no increased resistance to chloramphenicol, tetracycline, or kanamycin.

The activity and action of triclosan have been investigated in detail (Heath et al., 2001; Schweizer, 2001). Its possible use in food microbiology was alluded to earlier in this chapter, and it is thus relevant to examine briefly resistance to it in relation to antibiotic resistance. In essence, low-level antibiotic resistance in *E. coli* might be selected by triclosan; there is a possibility that triclosan usage might be associated with fluoroquinolone resistance in *P. aeruginosa* (Chuanchuen et al., 2001). There does not appear to be any relationship between antibiotic-resistant cocci and their (in)susceptibility to the bisphenol. A possible link has been established between mycobacterial (including *M. tuberculosis*) resistance to isoniazid and triclosan resistance.

E. coli O157:H7 contains the *mar* operon, and exposure to the antibiotic chloramphenicol induces *mar* mutants with cross-resistance conferred to ciprofloxacin and tetracycline. However, these *mar* mutants do not show cross-resistance to common food preservatives (benzoic acid, acetic acid, lactic acid) and thus appear to have no survival advantage in food compared with non-*mar* mutants (Golding et al., 2001).

In conclusion, whereas the possibility exists of a link between biocide usage and resistance and antibiotic resistance, the actual association (if any) is proving to be more elusive. Nevertheless, it is important that biocides in their various forms are used only when necessary and then in an appropriate manner.

STRESS ADAPTATION

The ability of bacteria and other microorganisms to adapt to chemical or other applied stress has long been known. In his classical text, Hinshelwood (1946) described the action of drugs on cells and the adaptation of cells to resist drug action. The term “drug” as used by him referred to antibiotic-type agents (sulphonamides) as well as to biocidal-type ones as exemplified by proflavine, propamidine, and crystal violet. The test organism was *Bacterium lactis aerogenes*. It was pointed out that adaptation, once acquired, was sometimes retained and sometimes lost (often rapidly, often slowly) when cells were subcultured repeatedly in the absence of a drug. These observations have been amply confirmed by more recent studies with a wider range of organisms and of biocides (Russell, 1999b). This has obvious implications in terms of the clinical and possibly domestic environments, in the food industry where disinfectants are used, and in the preservation of food, cosmetic, pharmaceutical, and possibly other types of products (Chapman et al., 1998). Additionally “cross-adaptation” (Hinshelwood, 1946) to chemically related biocides occurs as would be expected and occasionally to chemically unrelated ones. Again, ideas and results put forward for discussion over half a century ago have reemerged with the current emphasis on and debate about resistance to biocides, its significance and possible cross-resistance to other nonantibiotic compounds, and to antibiotics themselves. An interesting offshoot, and one that needs to be investigated more widely, is the finding that biocides, on the basis of high MIC values, that are inactive against a particular organism can, at low concentrations, prolong the lag phases of that organism in a nutrient medium at optimum growth temperature. This is then followed by a period of reduced growth and then by normal growth. This “adaptation to stress” is an interesting phenomenon, and the underlying reason could be instructive.

Gould (1989) pointed out that vegetative bacterial cells exposed to environmental stresses reacted homeostatically by adapting to the stress in a variety of ways. These included the activation and expression of new groups of genes following exposure to irradiation, osmotic shock, oxidative stress, or starvation. Heat-shock proteins are an obvious consequence, and as pointed out later, peroxide-inducible proteins show some degree of overlapping. Rowbury (1998) has suggested that secreted extracellular induction components could be involved in inducing resistance responses by effectively warning bacteria of impending stress. It would be interesting to conduct experiments with a range of biocides to determine the significance of such “alarmones.”

Pitt (1974) demonstrated that growth of *Zygosaccharomyces* (formerly *Saccharomyces*) *bailii* in the presence of subinhibitory concentrations of a preservative markedly increased its resistance to higher concentrations of that preservative. It is interesting in the light of subsequent observations, described later in this chapter, that higher resistance levels were found in the presence of glucose. Warth (1977) subsequently found that starved cells of this organism concentrated organic acids intracellularly, whereas in the presence of glucose this intracellular concentration was reduced considerably but could be overcome by means of metabolic inhibitors. From this, it is clear that the organism possessed an inducible preservative elimination (efflux) system (Warth, 1985, 1986, 1988). Organic acids, but not methyl paraben, are continuously removed from the cell (Warth, 1989a,b,c). Benzoate in energized cells is considered to be eliminated by flowing down the electrochemical gradient (Warth, 1988), and a similar model may account for the resistance of acid-tolerant yeasts to acetate (Moon, 1983). An alternative explanation put forward by Cole and Keenan (1987) envisages that resistance of *Z. bailii* is not the consequence of a simple internal pH (pH_i) independent extrusion pumping mechanism but rather that it relates to the ability of cells to tolerate

significant decreases in pH_i . The significance of this is the reestablishment of a compensatory "normal" pH_i value.

During bacterial sporulation, there is a shift in the activity of several hundred genes (with genes unique for sporulation being switched on) as well as in sigma factors, the substitution of which may alter the patterns of gene expression (Losick and Stragier, 1992; Kaiser and Loswick, 1993). Specific sigma factor M, σ^M , is necessary for the resistance of vegetative cells and outgrowing spores of *B. subtilis* to salt or thermal stress (Horsburgh and Moir, 1999).

When nonsporulating bacterial cells are subjected to nutrient limitation, growth rate decreases with rapid metabolic adjustment. One response, the stringent response, is mediated by an accumulation of ppGpp (guanosine 3¹-diphosphate-5¹-diphosphate, guanosine tetraphosphate), a product of the *rel* gene. This novel nucleoside occurs in response to amino acid deprivation (Sarubbi et al., 1988). There is a reduction in the rate of ribosomal RNA (rRNA) and phospholipid accumulation and a general stimulation of amino acid synthesis with binding of uncharged transfer RNA (tRNA), as opposed to aminoacyl-tRNA, to ribosomes. In cells lacking the stringent response (*rel*) as a result of *relA* mutation, there is an accumulation of rRNA. Studies with *E. coli* have demonstrated that growth rate is an inverse linear function of intracellular ppGpp accumulation, which regulates gene expression in a wide variety of cellular functions. When *E. coli* is subject to environmental stress, such as nutrient or amino acid deprivation (as mentioned earlier) or to antimicrobial agents, growth rate decreases and gene expression is markedly altered. This is essential for long-term cell survival and is partly mediated by alternative sigma factors such as σ^s encoded the *rpoS* gene (Whitehead et al., 1998; Greenaway and England, 1999a,b). The synthesis of σ^s can be activated by ppGpp by positively regulating *rpoS* expression.

The sensitivity of *E. coli* to antibiotics and biocides depends on their ability to produce ppGpp. Thus in *E. coli* 1648 (ppGpp positive), the cells are more resistant than isogenic mutants that have an impaired ability to produce ppGpp. An isothiazolone at sub-MIC level caused rapid changes in intracellular ppGpp concentration in exponentially growing cultures of *P. fluorescens* and especially in *P. aeruginosa* PAO1. *E. coli* strains varying in their ability to produce σ^s showed difference in susceptibility to some antibiotics and an isothiazolone. Thus *E. coli* RH90 (*rpoS*; Tn 10), an insertional mutant of strain MC4100, was unable to produce σ^s and was some eight times more sensitive than the parent strain (Greenway and England, 1999a,b).

Many aerobic bacteria have developed intrinsic defense mechanisms that confer tolerance to stress by peroxide, in particular hydrogen peroxide. The oxidative stress or SOS response has been described in *E. coli* and *Salmonella* and involves the production of neutralizing enzymes such as catalases, peroxidases, and glutathione reductase to prevent cellular damage and of exonucleases to repair lesions in DNA (Demple, 1991; Demple and Harrison, 1994; Storz and Altuvia, 1994). Increased tolerance is achieved by pretreatment with a subinhibitory dose of hydrogen peroxide, which induces a series of proteins. Many of these are under the control of a sensor/regulator protein (OxyR), including glutathione reductase and catalase (Demple and Halbrook, 1983; Winquist et al., 1984). Cross-resistance has been reported to heat, ethanol, and hypochlorous acid (Morgan et al., 1986; Chesney et al., 1996; Dukan and Touati, 1996). Peroxide-inducible proteins overlap with other stress proteins such as heat-shock ones (Morgan et al., 1986).

The oxidative stress response has been less extensively studied in Gram-positive bacteria, but *Bacillus* tolerance to hydrogen peroxide varies during the growth phase (Dowds et al., 1987). It is important to add, however, that the level of increased tolerance to hydrogen peroxide during the oxidative stress response may be insufficient to protect bacteria against a concentration (>3%) of peroxide generally used for antiseptic or disinfectant purposes (Hartford and Dowds, 1994).

Multidrug resistance (MDR) is a term used to describe resistance mechanisms used by genes that form part of the normal cell genome (George, 1996). MDR is a serious problem in enteric and other Gram-negative bacteria. The genes are activated by induction or mutation caused by some type of stress. Because the genes are distributed ubiquitously, genetic transfer is not needed. Several examples of MDR systems are known in which an operon or gene is associated with changes in

antibiotic or antimicrobial sensitivity. They include (1) mutations at an Acr locus in the Acr system rendering *E. coli* more sensitive to hydrophobic antibiotics dyes and detergents, (2) overexpression of the RobA protein in *E. coli* by the *robA* gene as a consequence of which multiple antibiotic and heavy metal resistance is conferred, and (3) the MarA protein that controls a set of genes (*mar* and *soxRS*) that encode resistance to antibiotics and superoxide-generating agents.

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21 Methods for Activity Assay and Evaluation of Results

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CONTENTS

Factors that Affect Antimicrobial Activity	660
Current Methodology	661
<i>In Vitro</i> Methods	663
Agar Diffusion.....	663
Dilution Methods.....	664
Gradient Plates	666
Wedge System.....	666
Spiral Plating.....	667
Automated Methods	668
Descriptive Methods	670
Combined Antimicrobial Systems	672
Application Studies	674
Interpretation of Results.....	675
Acknowledgments	677
References	677

Hundreds of compounds that are proven or potential antimicrobials for use in foods are discussed in this book. Unfortunately, there have been nearly as many methods used for determining antimicrobial effectiveness as there are compounds. This situation leads to several difficulties comparing results from different laboratories, determining antimicrobial effectiveness, establishing minimal inhibitory concentrations, and evaluating antimicrobial spectrum, among others. In addition, it is often difficult to determine the potential success of antimicrobials in a food or foods because of the use of methods that are inappropriate or lack relevance. Davidson and Parish (1989) reviewed the status of, and made recommendations concerning, methods for testing the effectiveness of food antimicrobials. They suggested that more uniformity and standardization should be instituted in testing food antimicrobials. Since that time, researchers have made some improvements in testing methods for food antimicrobials. The purpose of this chapter is to complement information presented in this book on antimicrobial effectiveness by suggesting methods that could be used in future studies. The subjects presented include factors affecting the activity of antimicrobials, methods for evaluating the effectiveness of single and multiple antimicrobials, and techniques for interpreting data gathered in testing. It is hoped that this information will be of benefit to academic, industrial, and regulatory researchers in producing data that are comparable among laboratories and that have relevance to applications of antimicrobials to foods.

FACTORS THAT AFFECT ANTIMICROBIAL ACTIVITY

In considering the testing of antimicrobials, the first issue that reasonably presents itself is as follows: what is the purpose of such testing? For antimicrobial agents in foods, it is difficult to answer this question in a simple and direct form because of the many and diverse foods to which they are added and the several possible antimicrobial agents that are being evaluated. The principle of all antimicrobial testing is to assess the degree of efficiency inhibiting or inactivating a selected range of organisms under specified conditions. All methods used to evaluate antimicrobial efficacy are influenced by factors that can affect the apparent activity of a compound. Factors inherent in the test microorganism, the antimicrobial agent, the test medium, and/or the test procedure itself may alter results such that an efficacious compound is judged ineffective, or vice versa. Additionally, a compound could be efficacious under the test conditions used but not provide adequate activity in the target food item under practical conditions. It is highly desirable, but often impractical or logistically impossible, to control all variables that could influence test results. It is therefore necessary to identify quantitative factors capable of influencing results. Table 21.1 highlights several factors that may influence the activity of antimicrobial agents.

One of the more important factors is the inherent nature of the specific test organism. Individual strains of a species often demonstrate genotypes and phenotypes different from the type strain. For instance, development and detection of antibiotic resistance by specific bacterial strains are well documented (Tenover, 1986). The microorganism(s) selected for use in the antimicrobial test method should adequately reflect the microflora of the target food item. In deciding on the type of microorganism to be used in any antimicrobial test, due attention is always given to the purpose for which the antimicrobial is required. Having selected a culture for a particular purpose, it should be immediately preserved so as to always have an available standard culture. If kept in continuous culture, it is not unknown for a microorganism to change its characteristics somewhat and for its resistance to vary. This is likely to occur even under the most carefully controlled cultural conditions, and in such circumstances the only solution is to revert to the original culture. All microorganisms have an optimum growth temperature, and they will grow over a reasonably wide range either side of this temperature. It is self-evident, however, that changes in temperature must affect the metabolic

TABLE 21.1
Selected Factors That May Affect Antimicrobial Activity

Test microorganism
Strain
Inoculum size
Cell physiology
Culture medium in which the organism is grown
Antimicrobial agent
Interaction of the test compound with components of the medium
Partial partition coefficient
Test medium
pH
Water activity
Redox potential
Test procedure
Incubation conditions
Oxygen pressure
Atmospheric CO ₂ concentration
Incubation temperature
Equipment variability

and growth rate of the organism and might even cause some change in the metabolic activity. This would inevitably lead to an alteration in the resistance of the culture and so affect the levels of response in a test. Sudden changes in the incubation temperature can also dramatically affect microbial viability.

The size and physiologic state of the inoculum are critical and must be well controlled. Piddock (1990) emphasized the critical nature of inoculum size in antibiotic susceptibility testing for the broth dilution and agar dilution techniques. Inocula that are too concentrated or too dilute can result in false-resistant or false-sensitive results, respectively. Research is needed to determine appropriate cell concentrations for use in testing food-related compounds. The physicochemical configuration of the target food and proposed shelf life affect inoculum preparation procedures and cell concentration used in antimicrobial testing. Cell physiology can also affect antimicrobial testing results. It is often assumed that actively growing vegetative cells (log-phase growth) are more sensitive to environmental stress caused by antimicrobial factors (chemicals, heating, water activity, and freezing, among others) than are stationary-phase vegetative cells or spores. Small variations in the age of the culture can give rise to large variations in its resistance during antimicrobial agent testing. Physiology may indeed play a role in determining cell sensitivity and resistance, but the effect of microbial growth phase on susceptibility to food antimicrobials is not well defined. The culture medium in which the organism is grown is also an important factor in influencing the characteristics of the microorganism. In several antimicrobial tests, it plays a dual role, first in maintaining the culture prior to its use in the test and second in providing the means of recovery and growth after treatment in the test.

Inherent factors of the test medium and test method have definite effects on cell sensitivity or resistance response. It is well documented that the pH of the medium affects the antimicrobial activity of such compounds as benzoic and sorbic acids. The antimicrobial action of these compounds is the result of the concentrations of the undissociated acid form, which is dependent on the pH of the medium. Water activity, partial oxygen pressure, atmospheric CO₂ concentration, redox potential, incubation temperature, and partition coefficient can also affect the physicochemical properties of specific antimicrobial compounds.

Other less obvious factors that can influence test results include interaction of the test compound with components of the medium, combined effects of factors that give the appearance of increased or decreased susceptibility, equipment variability, limitations of the plating method used for enumeration, purity of water, and analyst errors. For example, in agar diffusion techniques, the overall negative charge of agar resulting from sulfate groups may slow the diffusion rate of cationic antimicrobial agents, thereby affecting expected activity patterns (Piddock, 1990).

CURRENT METHODOLOGY

One of the first methods for evaluating the activity of disinfectants was developed by Robert Koch in 1881 (Block, 1983; Crémieux and Fleurette, 1983). He immersed silk threads in culture fluid containing *Bacillus anthracis* spores and exposed the dried threads to disinfectant for varying periods. He demonstrated that mercuric chloride was highly effective against the spores but that neither carbolic acid (phenol) nor ethanol had significant antimicrobial effects. Koch was probably the first to distinguish between bacteriostasis and lethality (Block, 1983). In 1897, Kronig and Paul noted that the antimicrobial activity of disinfectants could be compared only under controlled conditions and that kill rate was dependent on concentration and temperature (Block, 1983). Rideal and Walker used the principles of Kronig and Paul to develop the phenol coefficient method in 1903 (Crémieux and Fleurette, 1983). The novelty of their method was standardization of components, including microorganisms, recovery medium, disinfectant stock solution, and test methods. Although this method had many deficiencies, it continues to be used today with modifications (Beloian, 1990).

TABLE 21.2
Methods for Testing the Efficacy of Food Antimicrobials

In vitro, Screening, or Exploratory Tests

Endpoint Methods

Agar diffusion
Agar and broth dilution
Gradient plates
Spiral plating
Sanitizer and disinfectant tests

Descriptive Methods

Turbidimetric assays
Inhibition curves

Applied Tests

Endpoint methods
Inhibition curves

Combined Antimicrobials Tests

Agar diffusion
Dilution assays
Inhibition curves

Source: Adapted from Davidson and Parish (1989) and López-Malo et al. (2000a).

With the discovery of penicillin by Fleming in 1929, methods for determining the antimicrobial activity of antibiotics were developed. Assay methods were developed in the 1940s that involved diffusion of compounds in agar form wells or paper disks (Pidcock, 1990). At the same time, methods for determination of minimum inhibitory concentrations (MICs) using broth or agar dilution were developed. In 1966, Bauer et al. proposed a disk susceptibility method that had standardized procedures. Today, in the United States, the National Center for Clinical Laboratory Standards (NCCLS) publishes standardized procedures for agar diffusion and dilution assays for the activity of antibiotics and the bactericidal activity of antimicrobial agents (NCCLS, 1999, 2002). The available documents published by NCCLS include “Performance standards for antimicrobial disk susceptibility test” and “Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically,” among others.

Methods for evaluating the effectiveness of food-related antimicrobials have been in existence nearly as long as those for disinfectants and antibiotics. For example, Walton et al. (1936) determined the antimicrobial effect of vapors of crushed garlic against *Mycobacterium* species, *Escherichia coli*, *Serratia marcescens*, and *Bacillus subtilis*. In their procedure, crushed garlic was placed on the lid of a Petri dish and the bottom of the dish containing nutrient medium was inverted over the top. The vapors of the garlic were allowed to penetrate into the agar for varying periods. Following exposure, the medium with the test microorganism was incubated to determine inhibition. Antimicrobial effectiveness was related to time of exposure of the medium to the garlic vapors. This particular procedure was unique; most methods for evaluating the activity of food antimicrobials have been adopted directly or with modifications for other disciplines.

The classification of some of the most used methods to evaluate the antimicrobial efficiency in foods is shown in Table 21.2. Screening or *in vitro* tests (generally made in laboratory media or in model systems) provide preliminary information about the potential antimicrobial activity. The

endpoint tests, in which the microorganisms are challenged for an arbitrary period of incubation time, give qualitative information about approximate effective concentration. The descriptive screening methods, in which the microorganisms' growth is analyzed over time, give quantitative information about the growth dynamics. Finally, in applied tests, the antimicrobial is applied to the actual food and the results allow evaluation of some factors that can affect the efficacy of the natural antimicrobial (Davidson and Parish, 1989; López-Malo et al., 2000a). Current methods used to evaluate the efficacy of food antimicrobials may be divided into *in vitro* and application methods. *In vitro* methods include any test in which the compound is not applied to a product under use conditions. These methods can provide only preliminary information to determine the potential usefulness of the test compound in a food. Application methods are those in which an antimicrobial is applied directly to a food product to determine its effects on natural microflora or an inoculated microorganism.

In vitro methods include endpoint and descriptive analyses. Endpoint methods are those in which a microorganism is challenged for an arbitrary period and the results reflect the inhibitory power of a compound only for the time specified. In descriptive methods, periodic sampling is carried out to determine changes in viable cell numbers over time. Both these methods may be used to determine the effectiveness of antimicrobial combinations.

***IN VITRO* METHODS**

Agar Diffusion

The agar diffusion method (Acar and Goldstein, 1986; Piddock, 1990; NCCLS, 1999, 2002) has probably been the most widely used method for determination of antimicrobial activity throughout history. In this test, antimicrobial compound is added to an agar plate on a paper disk or in a well (Figure 21.1 and 21.2). The compound diffuses through the agar, resulting in a concentration gradient that is inversely proportional to the distance from the disk or well. Degree of inhibition, which is indicated by a zone of no growth around the disk or well, is dependent on the rate of diffusion of the compound and cell growth (Barry, 1986). Therefore, the antimicrobial evaluated should not be highly hydrophobic because the compound will not diffuse and little or no inhibition will be detected. The test microorganism selected must also grow rapidly and uniformly. Slow-growing strains produce large zones of inhibition, and vice versa (Piddock, 1990). This method should not be used for anaerobic microorganisms.

To run the test, Petri dishes are prepared to contain a nonselective medium at a depth of approximately 4 mm. The medium is surface inoculated with a suspension containing approximately log 6.0 colony-forming units (CFU)/ml of the test microorganism (Piddock, 1990). Known concentrations of antimicrobial are then added to filter paper disks (6 mm diameter), which are dried and placed on the surface of the previously inoculated plate (Figure 21.1). Alternatively, the antimicrobial may be added to wells cut in the agar with a sterile cork borer (Figure 21.2). Plates are incubated under optimum conditions for the test microorganism for 16 to 24 hours (NCCLS, 1999, 2002). Following incubation, plates are examined for zones of no growth indicated by halos around the disks (Figure 21.1). A control test microorganism with known susceptibility to the antimicrobial should be included.

The results of the agar diffusion test are generally qualitative. The susceptibility of the test microorganism is related to inhibition zone size in millimeters. Microorganisms are termed susceptible when the zone is >30 to 35 mm in diameter, intermediate with a zone of 20 to 30 mm, or resistant with a zone of <15 to 20 mm (Piddock, 1990). Determination of MIC is possible with this technique, but other methods are better.

A considerable amount of research has been done on the effectiveness of biologically derived antimicrobials, primarily bacteriocins produced by the lactic acid bacteria. Several methods related to the agar diffusion method are available for determining the activity of these compounds, including

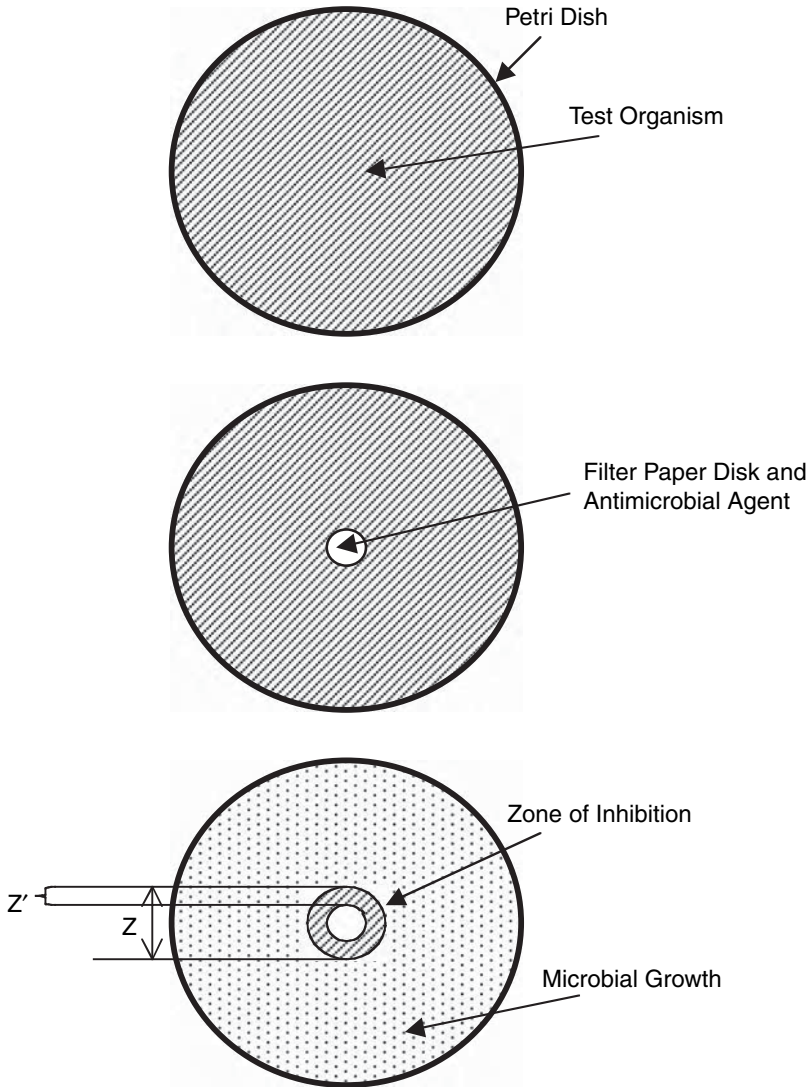


FIGURE 21.1 Determination of the “zone of inhibition” by the filter paper disk diffusion method.

the flip-streak and flip-spot methods (Kekessy and Piguet, 1970) and spot-on-lawn method. In studies by Harris et al. (1989) and Lewus and Montville (1991), the agar diffusion assay using wells was found to give a large number of false-negative results. Nisin activity is determined following the well diffusion assay described by Tramer and Fowler (1964) using an indicator strain (*Micrococcus luteus* ATCC 10420). Bacteriocin activity can be estimated from the size of the inhibition zones produced by the diffusion of the bacteriocin in the confluent lawns of sensitive bacteria (Montville et al., 2001).

Dilution Methods

Agar and broth dilution assays are generally used when quantitative data are desired, to determine whether an antimicrobial is lethal to a test microorganism, for microorganisms with a variable growth rate, and for anaerobic or microaerophilic microorganisms (Barry, 1986). These methods offer the food microbiologist a great deal of flexibility in determining the activity of a novel

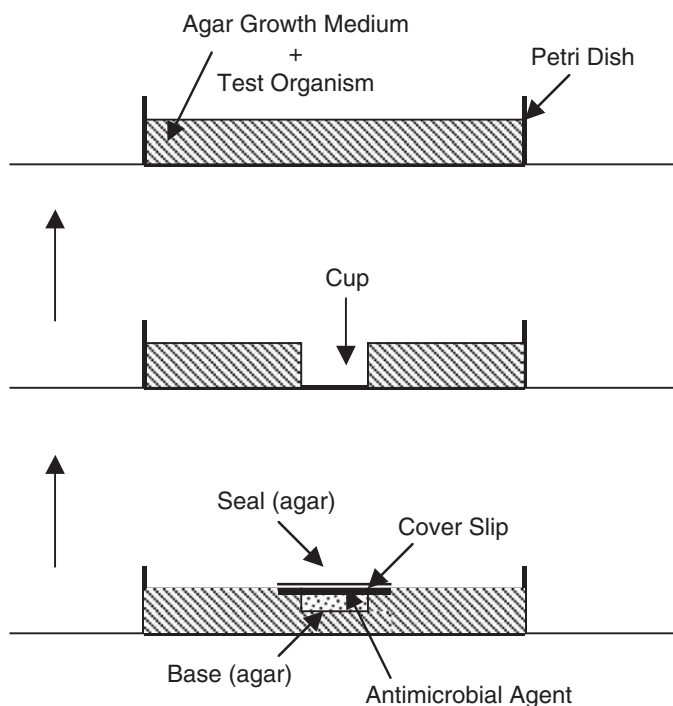


FIGURE 21.2 Agar cup method for the determination of antimicrobial activity of antimicrobial agents.

antimicrobial and/or the effect of environmental conditions on the growth of a microorganism in the presence of a known antimicrobial. In both methods a single statistic known as the MIC is generated. The MIC is generally defined as the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specified incubation period.

In the agar dilution method, a number of containers of molten nonselective agar are prepared. A single concentration of antimicrobial is then added to the agar in each container. The range of concentrations used is normally determined by trial and error. In clinical microbiology, doubling dilutions are used (e.g., 512, 256, 128, 64, and 32 $\mu\text{g/ml}$); however, any interval may be used as long as they are narrow enough to provide a realistic MIC. After addition of the antimicrobial to the agar, plates are poured and the agar is allowed to solidify. The test microorganism is diluted to around $\log 7.0$ CFU/ml and added to the plates in 1- to 2- μl spots (around $\log 4.0$ CFU per spot) (NCCLS, 1999, 2002). More than one species or strain may be used on one plate. A control plate, without added antimicrobial, should be prepared and inoculated to ensure adequate growth of the test microorganism. In addition, a control microorganism with known susceptibility should be included. Plates are incubated at the optimum temperature of the test microorganism for 16 to 24 hours (NCCLS, 1999, 2002). For some foodborne microorganisms, it may be desirable to incubate for longer periods under environmental stress conditions (Moir and Eyles, 1992). The MIC is considered the lowest concentration that completely inhibits growth (ignoring faint haze or a single colony). There are several advantages of the agar dilution method, including the ability to test a large number of strains at once, contamination is easily detected, and the medium can contain opaque materials (Barry, 1986).

In the broth dilution assay, an antimicrobial is serially diluted and a single concentration is added to a culture tube of nonselective broth medium. The concentrations are selected in the same manner as for the agar dilution method. Tubes (usually 1- to 10-ml total volume) are inoculated to contain approximately $\log 5.7$ CFU/ml of the test microorganism (Thrupp, 1986; Piddock, 1990; NCCLS, 1999). As with the agar dilution assay, a control tube without antimicrobial should be

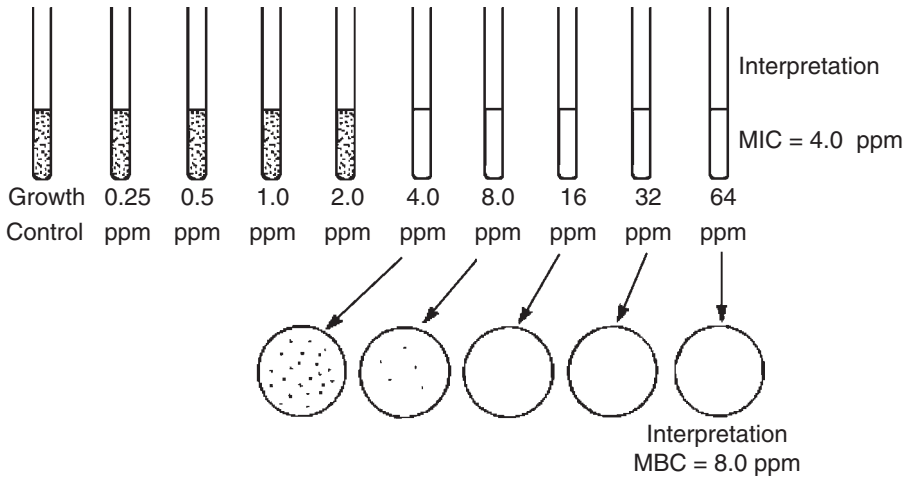


FIGURE 21.3 Determination of minimal inhibitory concentrations (MICs) and minimal bactericidal concentration (MBC) or minimal lethal concentrations (MLCs). The MIC is defined as the lowest concentration of antimicrobial concentration that results in no visible growth (turbidity). The MLC is the lowest concentration of antimicrobial that produces a 99.9% (10^3) reduction in organisms from the original inoculum of approximately 10^5 . (Adapted from Conte and Barriere, 1992.)

prepared and inoculated and a strain with known susceptibility should be included. Tubes are incubated for 16 to 24 hours at the optimum temperature of the test microorganism. As before, the incubation time may have to be adjusted depending on the environmental conditions being evaluated. The MIC is defined as the lowest concentration at which no growth occurs (absence of turbidity) in a nutrient medium following incubation.

The broth dilution assay may also be carried out on a microscale. In the microdilution broth assay, 50- to 100- μ l broth plus a single concentration of antimicrobial is added to the well of a microtiter plate. The inoculum is added at a level of approximately 4.7 CFU per well. The remainder of the assay is the same as the standard broth dilution assay.

As mentioned, broth dilution assays may be used to determine the lethality of the test microorganism. To establish lethality, the standard or microdilution broth assay must be carried out as described. Following incubation of the tubes or microtiter plates, 10 to 100 μ l medium from the last tubes or wells in which the microorganism exhibits growth and all tubes or wells showing no growth (no turbidity) are plated on a nonselective agar using the spread-plate method. The lowest concentration of antimicrobial that produces $\geq 99.9\%$ kill of the test microorganism is termed the minimum bactericidal concentration (MBC) or minimum lethal concentration (MLC) (NCCLS, 2002). Figure 21.3 presents as an example the tube dilution method determining the MIC as well as the MBC or MLC.

GRADIENT PLATES

Wedge System

Wedge plating is a subjective method of observing relative sensitivity to antimicrobial agents among several microorganisms on an agar medium. It is similar to the agar diffusion methods because the concentration of antimicrobial agent to which a microorganism is exposed depends on the ability of the agent to diffuse through agar.

In this technique, a known concentration of antimicrobial is aseptically added to a nonselective base agar medium, which is poured into a Petri dish with one side raised to create a slanted surface. Square Petri plates are often used for this method to create a uniform gradient. After hardening,

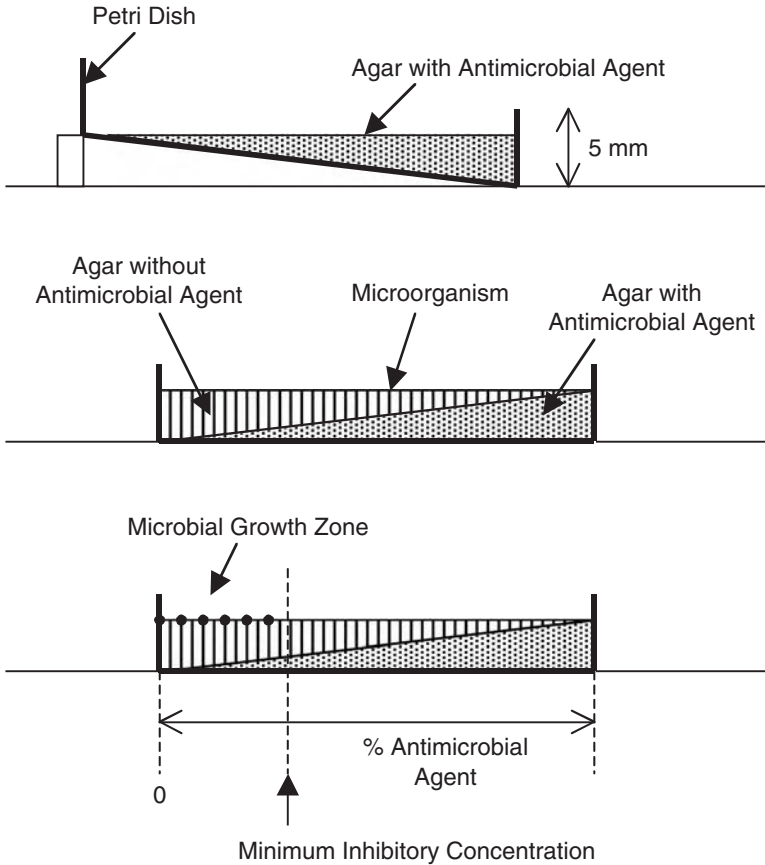


FIGURE 21.4 Gradient plate method for the determination of antimicrobial activity of antimicrobial agents.

the dish is placed on a flat surface and the base medium without added antimicrobial is poured over the surface of the slanted agar (Figure 21.4). The gradient is established depending on the rate at which the antimicrobial diffuses through the agar. Cells of known concentration from a broth culture are then streaked across the gradient surface. Control strains with known susceptibility or resistance to the test compound are streaked next to the test organism using broth cultures of the same cell concentration. Plates are incubated with an appropriate temperature and time regimen for the test microorganism. Growth suppression of the test organism is observed in relation to the control strains.

This method of antimicrobial testing can be semiquantitative if proper control strains of known sensitivity are selected for use. Such factors as incubation (exposure) time and inoculum preparation and size must be consistent for reproducible results. Bala and Marshall (1996) developed a double-gradient plating technique to evaluate the influence of NaCl and monolaurin on inhibition of *Listeria monocytogenes*.

Spiral Plating

The spiral plating system is used for enumeration of cells in microbial suspensions (Spiral Biotech, Inc., Norwood, MA); however, a spiral gradient endpoint (SGE) test for determination of MIC has been developed for antibiotics (Paton et al., 1990; Spiral Biotech, 2001; Hill, 1991) and could potentially be used to determine the MIC of antimicrobial compounds use in food systems (Schalkowsky, 1986).

The spiral plater dispenses a continuously decreasing volume of a known concentration of antimicrobial onto an agar medium surface in an Archimedes spiral pattern. This results in a gradient of the test compound with a high concentration near the center of the Petri plate. Cell suspensions of control and test organisms are radially streaked onto the surface of the plate from the outside edge to near the plate center. After appropriate incubation, the distance from the end of growth to where the antimicrobial deposition began (about 13 mm from the plate center) is measured and recorded. The distance is used to calculate a discrete MIC, as opposed to an MIC based on traditional incremental dilution methods (Paton et al., 1990). Observation of the growth to no-growth region provides a basis for measuring the MIC. Figure 21.5 presents the template used to read the end growth point and shows an example of how microbial growth is observed when using this technique. Using this methodology Astorga-Solari et al. (2001) reported that it was possible to evaluate the antimicrobial effectiveness of thymol, eugenol, potassium sorbate, and sodium benzoate to inhibit the growth of different bacteria (*Listeria innocua*, *Escherichia coli*, and *Lactobacillus* species). The SGE test correlates well with conventional dilution methods; however, there are few data regarding its use with food antimicrobials.

Automated Methods

Automated and semiautomated methods have been developed to provide rapid enumeration and increased endpoint accuracy with reduced technical error. Some systems were originally developed for rapid enumeration and identification but can also provide information regarding antimicrobial susceptibility testing. Piddock (1990) reviewed the use of automated methods for antimicrobial susceptibility testing. Specific methods addressed include the Autobac broth disk elution system, MS-2 Microbiology System, AMS (Automicrobic) system, and four microtiter tray tests (Touch-SCAN/AutoSCAN, Sceptor, Sensititre, and Micro-Coder). Thornsberry (1986) provides specific procedural information for the Autobac, MS-2, and MIC-2000 systems. These systems correlate well with conventional susceptibility testing; however, their use has been directed at clinical testing. Methods involving impedance, bioluminescence, pH changes, radiometry, and microcalorimetry have also been investigated (Piddock, 1990). As the field of rapid methods and automation develops, the boundaries between instrumentation and diagnostic tests began to merge. Instrumentation is now playing an important function in diagnostic systems, and the trend will continue (Fung, 2002). Instruments are needed to monitor changes in a population such as adenosine triphosphate (ATP) levels, specific enzymes, pH, electrical impedance, conductance and capacitance, generation of heat, radioactive carbon dioxide, and so on. It is important to note that for the information to be useful these parameters must be related to viable cell count of the same sample series.

In general, the larger the number of viable cells in the sample, the shorter the detection time of these systems. The assumption is that as the number of microorganisms increases in the sample, these physical, biophysical, and biochemical events will also increase accordingly. When a sample has 5 log or 6 log organisms/ml, detection time can be achieved in about 4 hours. Currently the following ATP instruments are available: Lumac (Landgraaf, the Netherlands), BioTrace (Plainsboro, NJ), Lightning (BioControl, Bellevue, WA), Hy-Lite (EM Science, Darmstadt, Germany), Charm 4000 (Charm Sciences, Malden, MA), Celsis system SURE (Cambridge, UK), Zylux (Maryville, TN), Profile 1 (New Horizon, Columbia, MD), and others.

Investigations of biocide efficacy by automated methods involving optical density measurements (e.g., using the recently published "Bioscreen" method) are complicated by the fact that a poor correlation often exists between the log reductions obtained using the automated method versus those obtained by the traditional plate count methods (Lambert and van der Ouderaa, 1999). It was assumed that the differences observed between the two methods were the result of the level of cell injury, which was masked by the optical density methods but which was recognized by the plate counts. Comparisons of log reductions following a disinfection test always showed the Bioscreen method to be overestimating the log reductions with respect to the plate counts. Lambert and

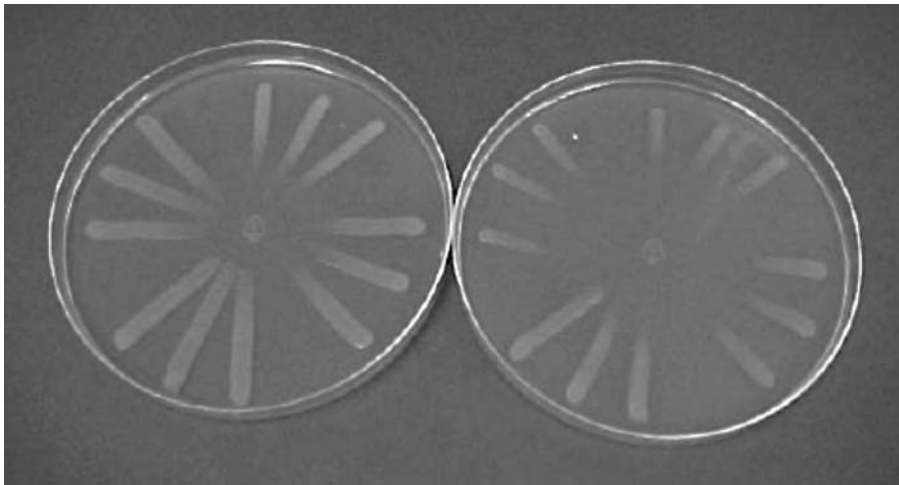
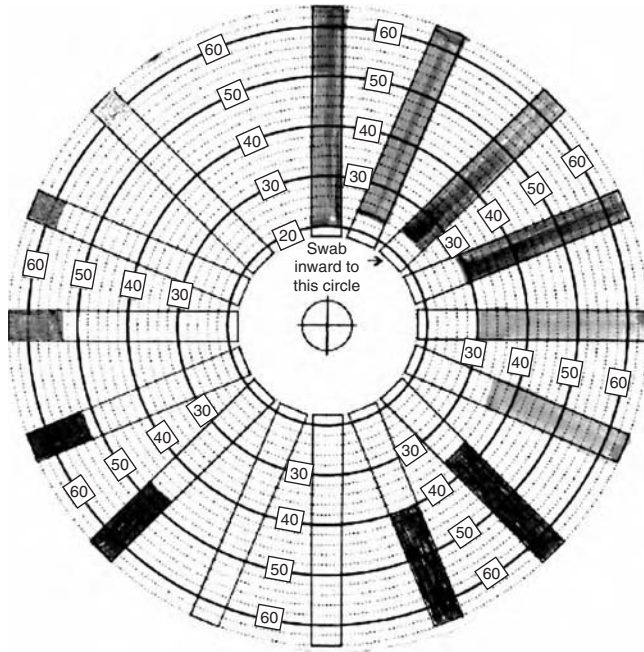


FIGURE 21.5 Spiral gradient endpoint template and *Listeria innocua* growth response to sodium benzoate (*left*) and potassium sorbate (*right*) deposited using this technique over solidified SPC agar.

van der Ouderaa (1999) reported a correlation between colony size on the plates and the Bioscreen results for a fixed disinfectant concentration, and contact time was found using image analysis. The results obtained also suggested that the observed colony area was dependent on the amount of injury incurred by a microbe during the disinfection process. A mathematic model of injury was developed that predicted the observed differences between the Bioscreen and the traditional plate method. The model further suggested a possible reason for biocidal lags (Lambert and van der Ouderaa, 1999).

As microorganisms grow and metabolize nutrients, large molecules change to smaller molecules in a liquid system and cause a change in electrical conductivity and resistance in the liquid as well as at the interface of electrodes. These changes can be expressed as impedance, conductance, and

capacitance changes. When a population of cells reaches about 5 log CFU/ml, it will cause a change of these parameters. Thus, when a food has a large initial population, the time to make this change will be shorter than a food that has a smaller initial population (Fung, 2002). The time the curve changes from the baseline and accelerates upward is the detection time of the test sample, which is inversely proportional to the initial concentration of microorganisms in the food. To use these methods a series of standard curves must be constructed by making viable cell counts of a series of foods with different initial concentration of cells and then measuring the resultant detection time. The Bactometer (bioMerieux, Hazelwood, MO) has been in use for many years to measure impedance changes in foods, water, cosmetics, and so on by microorganisms. Samples are placed in the wells of a 16-well module, which is then plugged into the incubator to start the monitoring sequence. As the cells reach the critical number (5 log to 6 log/ml), the change in impedance increases sharply and the monitor screen shows a slope similar to the log phase of a growth curve. The detection time can then be obtained to determine the initial population of the sample. A similar system called RABIT (Rapid Automated Bacterial Impedance Technique), marketed by Bioscience International (Bethesda, MD), is available for monitoring microbial activities in food and beverages. Instead of a 16-well module used in the Bactometer, individual tubes containing electrodes are used to house the food samples. The Malthus system (Crawley, U.K.) uses conductance changes of the fluid to indicate microbial growth. It generates conductance curves similar to impedance curves used in the Bactometer. It uses individual tubes for food samples. All these systems have been evaluated by various scientists in the past 10 to 15 years with satisfactory results (Fung, 2002). All have their advantages and disadvantages depending on the type of model system or food being analyzed. Research is needed to further evaluate automated methods for use with food antimicrobial systems.

DESCRIPTIVE METHODS

In endpoint methods, the concentration of an antimicrobial required to totally inhibit a microorganism is determined. These methods provide little information concerning the effects of an antimicrobial that are below the MIC. Certain antimicrobials may still cause an increased lag phase, reduced growth rate, or even initial lethality followed by growth. In food products, total inhibition of a pathogen of spoilage microorganism is not always required. An increased lag phase, especially under conditions of severe abuse, is often sufficient to protect the consumer. Therefore, to determine the effect of a compound on the growth (or death) kinetics of a microorganism, a descriptive method is required.

The most commonly used descriptive technique involves producing an inhibition curve with the colony count procedure. In clinical microbiology, these inhibition curves are known as "time-kill curves" (Schoenkecht et al., 1985; NCCLS, 2002). The procedure uses a nonselective broth medium (10 to 100 ml) to which is added a single concentration of antimicrobial. The concentration may be determined from the endpoint methods previously described. The test microorganism is diluted to achieve a final cell concentration of approximately log 5.7 CFU/ml in the test medium (NCCLS, 2002). If the effect of the compound on the lag phase is to be evaluated, a lower number (e.g., log 3 to 4 CFU/ml) can be used. The NCCLS (2002) recommends against inoculum levels $>\log 6$ CFU/ml because selection of resistant mutants may occur. A tube or flask without added antimicrobial should be prepared as a control. The medium is incubated at the optimum temperature of the test microorganism for up to 48 hours. The medium is sampled at appropriate times (e.g., 0, 2, 4, 8, 12, 24, and 48 hours) and the number of viable microorganisms is determined by the spread or pour plate method. Several responses of the test microorganism may be encountered in this type of analysis, including stationary-phase growth level suppression, lag-phase increase, decrease in the growth rate during log phase, and lethality (Davidson and Parish, 1989). In practice, a distinction is frequently drawn between a fungistatic or bactericidal action and a fungicidal or bactericidal action. This difference may not be completely justified because the former two differ from the latter in the death rate of the microorganisms. In the long term the effect of added

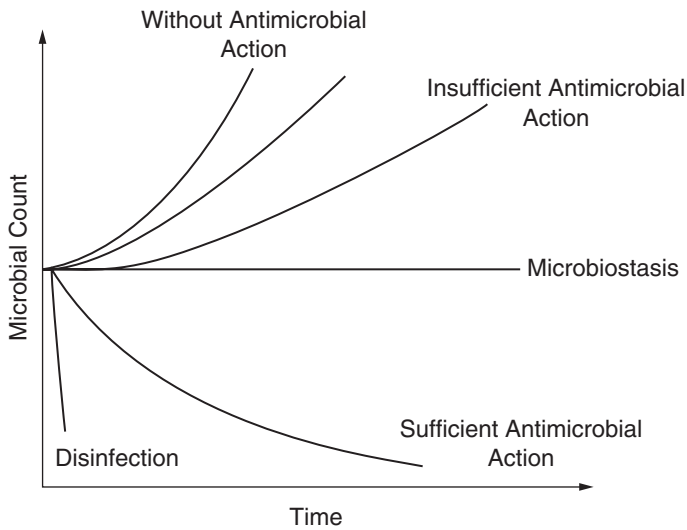


FIGURE 21.6 Effect of antimicrobial agent dose on microbial response. (Adapted from Lück and Jager, 1997.)

antimicrobial in food is either to inactivate (kill) the microorganisms or to allow them to grow despite its presence. The governing factor here is the dosage of antimicrobial (Figure 21.6).

Depending on the type of antimicrobial used, all the microorganisms are inactivated (killed) within days or weeks at the usual applied concentrations. This is where the difference exists between antimicrobials and disinfectants. The latter generally can be used only if the microbial count is drastically reduced within a very short period. Inhibition curves can be used to evaluate the antimicrobial action of several agents against bacteria, yeast, and molds. However, for molds their radial growth rate as well as the germination time (time to form a visible colony) often are used as measures of antimicrobial effectiveness (López-Malo et al., 1995, 1997, 1998). The inhibition curve methodology is the only technique that demonstrates lethality. This method is versatile but has some disadvantages, including that no single statistic is produced to compare such treatments as MIC. It is also labor intensive and expensive.

Progress made in modeling the growth kinetics of microorganisms (Zwietering et al., 1990, 1991; McMeekin et al., 1993; Whiting, 1993, 1995, 1997) should allow improved statistical analysis of growth-inhibition curves in the presence of food antimicrobials. More recently, probabilistic modeling has been used to determine growth/no-growth interface. Probabilistic models based on logistic regression analysis provide a useful way to describe the growth/no-growth boundary (Ratkowsky and Ross, 1995; Presser et al., 1998; López-Malo et al., 2000b), including the effect of selected antimicrobial concentrations. This methodology allows defining the antimicrobial concentration range necessary to inhibit growth under selected environmental conditions (Alzamora and López-Malo, 2002).

A second method for determining antimicrobial effectiveness over time is to measure turbidity increases with a spectrophotometer. A major disadvantage of this type of analysis is the sensitivity of the instrument. Spectrophotometers generally require log 6.0 to 7.0 CFU/ml for detection (Brock et al., 1984; Piddock, 1990). This may create a situation in which no growth (i.e., no absorbance increase) is observed when, in fact, undetectable growth is occurring at levels below log 5.0 CFU/ml. An erroneous interpretation of “lethality” could result (Davidson and Parish, 1989). A method of using turbidimetric analysis to determine infinite inhibitory concentrations (IIC, analogous to MIC) was developed by Marwan and Nagel (1986). In their procedure, the time for a microbial population to reach a specific turbidity in the presence of an antimicrobial was divided by the time for the

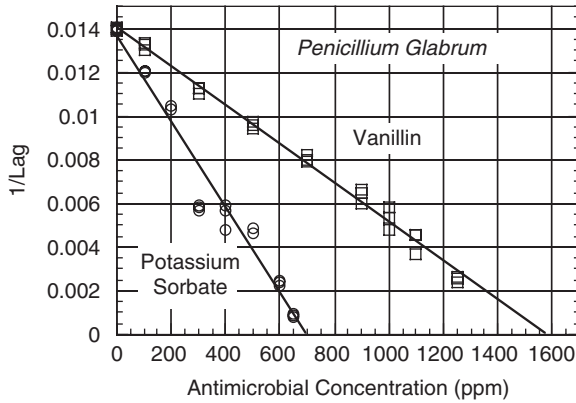


FIGURE 21.7 Relative effectiveness (inverse of lag time) of selected concentrations of vanillin and potassium sorbate on *Penicillium glabrum* inoculated in potato dextrose agar.

same population to reach the turbidity in the absence of the antimicrobial. This was termed the relative effectiveness (RE). When $1/RE$ was plotted versus concentration of the inhibitor, the response was linear. Marwan and Nagel determined that at $1/RE = 0$ (IIC), the concentration of antimicrobial would totally inhibit the test microorganism. As an example, the inverse of lag time of *Penicillium glabrum* inoculated in potato dextrose agar formulated with selected concentrations of vanillin and potassium sorbate is shown in Figure 21.7. The response was linear and the calculated inhibitory concentrations and infinite lag time, correspond to MICs determined using the agar dilution method. Lambert and Pearson (2000) described a test to obtain MIC using optical density (OD) determinations. The basis of the technique is the comparison of the area under the optical density versus time curve of the control (microorganism without antimicrobial) with the areas of the microbial curve with the test antimicrobial in several dilutions. The authors use 100-well plates and an OD automatic reader capable of giving an output signal based on a microbial growth criterion. As the amount of antimicrobial agent in a well increases, the effect on the growth is indicated by a reduction in the area under the curve. In general, plotting the inhibitor concentration on a logarithmic scale gives a characteristic sigmoid-shaped curve. The curve can be split into three principle regions: a region where the presence of the antimicrobial has no effect on the organisms relative to the control growth, a region where there is increasing inhibition of growth, and a region where there is no measurable growth relative to the control. This approach permits calculation of the MIC as the concentration above which no growth is observed relative to the control.

To summarize the use of *in vitro* methods, they should be used together: one endpoint and one descriptive method. The endpoint method helps to determine the approximate effective concentration, and the descriptive method evaluates the effect of a compound on growth over time.

COMBINED ANTIMICROBIAL SYSTEMS

Traditionally, it was common to use only one chemical antimicrobial agent in a food product for preservation purposes (Busta and Foegeding, 1983); however, in recent years, the use of combined agents in a single food system has become more frequent. The use of combined antimicrobial agents theoretically provides a greater spectrum of activity, with increased antimicrobial action against the pathogenic or spoilage organisms. It is thought that combined agents act on different species of a mixed microflora or act on different metabolic elements within similar species or strains. This theoretically results in improved microbial control over the use of one antimicrobial agent alone; however, actual proof of improved efficacy requires subjective interpretations. Although testing of combined antibiotics for clinical use is well studied and relatively well standardized

TABLE 21.3
Checkerboard Array in which Serial Dilutions of Two Antimicrobials Are Performed Using Concentrations Proportional to the MICs of the Antimicrobials Being Tested

2.0	2.0/0	2.0/0.06	2.0/0.12	2.0/0.25	2.0/0.50	2.0/1.0	2.0/2.0
1.0	1.0/0	1.0/0.06	1.0/0.12	1.0/0.25	1.0/0.50	1.0/1.0	1.0/2.0
0.50	0.50/0	0.50/0.06	0.50/0.12	0.50/0.25	0.50/0.50	0.50/1.0	0.50/2.0
0.25	0.25/0	0.25/0.06	0.25/0.12	0.25/0.25	0.25/0.50	0.25/1.0	0.25/2.0
0.12	0.12/0	0.12/0.06	0.12/0.12	0.12/0.25	0.12/0.50	0.12/1.0	0.12/2.0
0.06	0.06/0	0.06/0.06	0.06/0.12	0.06/0.25	0.06/0.50	0.06/1.0	0.06/2.0
0	0/0	0/0.06	0/0.12	0/0.25	0/0.50	0/1.0	0/2.0
	0	0.06	0.12	0.25	0.50	1.0	2.0

(Barry, 1976; Krogstad and Moellering, 1986; Eliopoulos and Moellering, 1991), application of such methodology to antimicrobials in food systems is less well developed (Davidson and Parish, 1989).

Methods of testing combined antimicrobials usually involve agar diffusion, agar or broth dilution, or inhibition (time-kill) curves. Agar diffusion is a simple qualitative method that often requires subjective interpretation of inhibition zone shapes to determine the efficacy of combined antimicrobials (Barry, 1976). This method provides a measure of microbial growth inhibition but does not address biocidal activity. Dilution methods yield quantitative data and are most often conducted with various combined concentrations of two antimicrobials arranged in a “checkerboard” array. The checkerboard method is the technique used most frequently to assess antimicrobial combinations *in vitro*, presumably because (1) its rationale is easy to understand, (2) the mathematics necessary to calculate and interpret the results are simple, (3) it can readily be performed in the laboratory using microdilution systems, and (4) it has been the technique most frequently used in studies that have suggested an advantage of synergistic interactions of antibiotics in clinical treatments (Eliopoulos and Moellering, 1991). The term checkerboard refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentrations equal to, above, and below their MIC (Table 21.3). Traditional clinical dilution testing uses twofold dilutions of test compounds, but testing of food antimicrobials is often conducted with alternate dilution schemes (Rehm, 1959; Davidson and Parish, 1989; Eliopoulos and Moellering, 1991). Inhibition or time-kill curves can also be used for evaluation of combined antimicrobials (Krogstad and Moellering, 1986; Eliopoulos and Moellering, 1991). The advantage of this test is that rates of lethality can be determined; however, repetitive sampling limits the number of combinations that can be assayed at any one time.

Combined studies are conducted to determine if specific types of interactions occur between the two combined antimicrobials. Traditionally, the terms “additive,” “antagonistic,” and “synergistic” were used to describe possible antimicrobial interactions (Table 21.4). Garrett (1958) suggested the terms “additivity” and “equivalence” to describe combination interactions; however, our discussion is limited to the more traditional descriptors.

Additivity occurs when two combined antimicrobials give results that are equivalent to the sum of each antimicrobial acting independently. There is no enhancement or reduction in overall efficacy for the combined antimicrobials compared to the individual results; this is also sometimes referred to as “indifference” (Krogstad and Moellering, 1986; Eliopoulos and Moellering, 1991). Antagonism refers to reduce efficacy of the combined agents compared to the sum of the individual results. Synergism is an increase or enhancement of overall antimicrobial activity when two agents are combined compared to the sum of individual results.

A conclusion of synergism must be approached with caution because it implies that a reduction in overall antimicrobial concentration might be achieved in a food system without a reduction in

TABLE 21.4
Quantitative Definitions of Results with Antimicrobial Combinations

Additivity	
The result with two antimicrobials is equal to the sum of the results for each of the antimicrobials used separately	$FIC_A + FIC_B = 1.0$
Antagonism	
The result with two antimicrobials is significantly less than the additive response	$FIC_A + FIC_B > 1.0$
Synergism	
The result with two antimicrobials is significantly greater than the additive response	$FIC_A + FIC_B < 1.0$
<i>Note:</i> FIC_A and FIC_B are the fractional inhibitory concentrations of antimicrobial A and B, respectively.	

efficacy. Gardner (1977) stated that true synergism is rare in relation to combined antibiotics. Other concerns about the misuse of the term “synergism” in relation to antimicrobials have been previously cited (Davidson and Parish, 1989; Garrett, 1958). Most commonly, additive interactions are misidentified as synergistic. A case in which an increase in antimicrobial activity is observed on the addition of a second compound to a food system does not necessarily constitute synergy. A conclusion of synergism requires that the overall efficacy of the combination be significantly greater than the sum of the efficacies of the individual compounds.

From a practical standpoint, processors must beware of the temptation to use combined antimicrobials without thorough scientific scrutiny. This is mainly because of possible antagonistic interactions that can reduce overall antimicrobial efficacy compared to the individual compounds used singly. Some food-related antimicrobial combinations that have been interpreted as antagonistic include sulfite and butylated hydroxyanisole, ethanol and butyl paraben, SO_2 and butyl paraben, SO_2 and benzoate, and SO_2 and sorbate (Parish and Carroll, 1988b; Davidson and Parish, 1989).

APPLICATION STUDIES

The primary purpose of *in vitro* methods is to determine the concentration of an antimicrobial necessary to inhibit a microorganism in a laboratory medium. However, once it has been determined that an antimicrobial is effective using *in vitro* methods, it is still necessary to apply the compound to a food system. Often, an antimicrobial that performed well in a microbiological medium is shown to have little or no effect in a food. This is the result of many interacting factors in a food, such as proteins, lipids, cations, binding to food components, inactivation by other additives, pH effects on antimicrobial stability and activity, uneven distribution in the food matrix, and poor solubility, among others.

Before actual application of an antimicrobial to a food, it may be useful to evaluate the effect of some component in a food system that may influence the effectiveness of the compounds, such as lipids, proteins, and divalent cations. Lipids may cause a decrease in activity of lipophilic compounds, and because many food antimicrobials have a hydrophobic character, there is invariably some reduction (Rico-Muñoz and Davidson, 1983). Proteins may cause binding of some compounds and reduce activity (Rico-Muñoz and Davidson, 1983). López-Malo et al. (1995) prepared fruit-based agars containing mango, papaya, pineapple, apple, and banana with up to 2000 ppm vanillin to assess the effect of fruit composition on the MIC to inhibit *Aspergillus flavus*, *A. niger*, *A. parasiticus*, and *A. ochraceus*. The small differences in protein and lipid composition between banana or mango and the other fruits increased vanillin MIC attributing the effect to binding of

the phenolic vanillin by protein. Divalent cations may affect the activity of some compounds by affecting the microorganism itself or by interacting with the antimicrobial (Rico-Muñoz and Davidson, 1984). Further information can be gained by using model systems that include a percentage of a food in a buffer or microbiological medium. For example, Dje et al. (1989) evaluated antimicrobials in model systems containing a 10% (wt/vol) suspension of raw chicken or frankfurters. They prepared the suspensions by homogenizing 25 g of product with 225 ml 0.1% peptone diluent. A homogeneous suspension of frankfurter was facilitated by adding 0.25% Tween 80 (sorbitan monooleate polyoxyethylene). In addition to determining the effect of food components on activity, this procedure allows better control of environmental conditions and natural microflora. In both preceding model systems, a modified broth dilution assay could be used to determine an MIC (endpoint method), or periodic sampling (descriptive method) could be run.

In applying an antimicrobial to a food, the microorganisms should include natural contaminants (bioburden) and the pathogen of interest. The incubation conditions should reflect normal storage and abuse. The success of the test may be through increased shelf life or reduced growth rate of a foodborne pathogen. Few food antimicrobials actually cause lethality of foodborne microorganisms at normal use concentrations. No standardized methods or application methods are available; however, a modification of the endpoint method or time-kill curve is possible. Although it is impractical to design uniform procedures for all products, as is done for screening methods, a good approach is to develop a uniform in-house procedure based on standardized procedures. Microbial challenge testing can be used to study the fate of pertinent microorganisms in foods formulated with antimicrobial agents as a way to assess product stability and safety (Tapia de Daza et al., 1995). Microbial challenge test has become an established technique within the food industry to simulate what can happen to a product during processing, distribution, and subsequent handling, following inoculation with more relevant microorganisms and further holding under controlled conditions (Notermans et al., 1993). This procedure can be used to verify antimicrobial agent effectiveness in foods.

INTERPRETATION OF RESULTS

The most difficult aspects of antimicrobial testing are appropriate data analysis and interpretation. Erroneous research conclusions from improper data interpretation can ultimately lead to industrial losses when practiced commercially. It is therefore necessary for data analysis to be conducted with great care.

Agar diffusion techniques produce quantitative zone diameter data. These results indicate relative microbial inhibition among a group of antimicrobials or test microorganisms. In antibiotic disk testing, the inhibition zone diameter of a specific test organism and antibiotic is compared to zone sizes produced with control organisms (Figure 21.1). The method correlates inhibition zone size with the concentration of the antimicrobial agent (Conte and Barriere, 1992). It must be noted that inhibition zone sizes are influenced by a number of factors, including inoculum density, agar depth, agar medium composition, incubation time, and temperature (Barry, 1986).

Combined antimicrobial testing using the agar diffusion method produces inhibition zones that are interpreted depending on their shape. This technique makes use of paper strips or disks impregnated with a specific quantity of antimicrobial agent and placed on an agar surface seeded with the test microorganism. This method is more qualitative than dilution testing but is useful as an endpoint method for large numbers of preservative combinations.

As previously discussed, dilution testing results in an MIC that is the first concentration of diluted compound that inhibits the test organism for a specific period. This reflects the minimum concentration of a compound necessary to inhibit growth of an organism under specific conditions. It should be noted that the MIC could vary depending on factors mentioned earlier.

Interpretation of dilution results for combined testing is usually conducted with isobolograms. An isobologram may be thought of as an array of differing concentrations of two compounds,

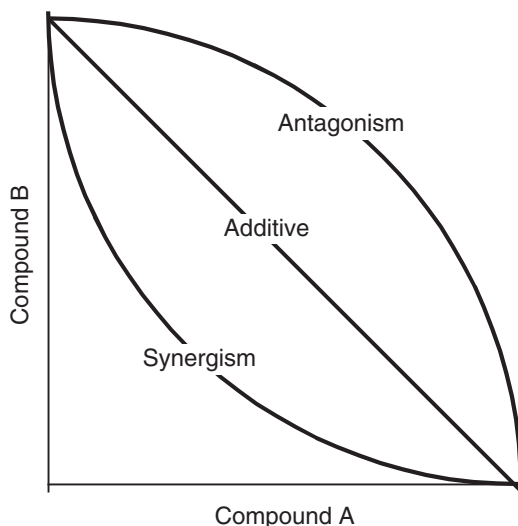


FIGURE 21.8 Interpretation of time-kill curves for combined antimicrobials.

where one compound ranges from lowest to highest concentration on the x-axis and the other on the y-axis (Figure 21.8). All possible permutations of combined concentrations are reflected within the array. If those concentrations that inhibit the growth of the test organism fall on an approximately straight line that connects the individual MIC on the x and y axes, the combined effect is additive. Deviation of linearity to the left or right of the additive line is interpreted as synergism or antagonism, respectively.

Isobologram construction can be simplified using fractional inhibitory concentrations (FICs), which are MICs normalized to unit. The FIC is the concentration of a compound needed to inhibit growth (expressed as a fraction of its MIC) when combined with a known amount of a second antimicrobial compound. It is calculated as the ratio of the MIC of a compound when combined with a second compound divided by the MIC of the first compound alone. Additive, synergistic, or antagonistic interactions are interpreted as with an MIC isobologram. For example, if the MIC for sorbate (under specific test conditions) is determined to be 200 ppm and the MIC for sorbate when combined with X ppm of propylparaben is 120 ppm, then the FIC for sorbate in the presence of X ppm propylparaben is $120/200 = 0.6$.

The FIC of two compounds in an inhibitory combination may be added to give a total FIC_{index} . If the FIC of propylparaben in the previous example was determined to be 0.5, then FIC_{index} would be $FIC(\text{sorbate}) + FIC(\text{propylparaben}) = 0.6 + 0.5 = 1.1$. An FIC_{index} near 1 indicates additivity, whereas <1 indicates synergy and >1 indicates antagonism (Table 21.4). The degree to which a result must be less than or greater than 1 to indicate synergism or antagonism is a matter of interpretation. Squires and Cleeland (1985) proposed that additive results are indicated by FIC_{index} between 0.5 and 2.0 for antibiotic testing. Synergism and antagonism are indicated by results <0.5 and >2.0 , respectively. Research is needed to provide a database for proper interpretation of FICs and of the FIC_{index} in relation to food antimicrobial systems. Data interpretation must be conducted conditionally and will depend on a number of variables, such as specific test conditions, microbial strain, and target food system. It should be noted that interpretations might also vary depending on the specific concentrations of each antimicrobial used in combination. Parish and Carroll (1988a) observed additivity between SO_2 and either sorbate or butylparaben when the inhibitory concentration contained less than 0.25 FIC of SO_2 ; however, the same combination at higher SO_2 FIC indicated antagonistic results. Rehm (1959) observed similar anomalies when sodium sulfite was combined with formate or borate. Branen and Davidson (2003) demonstrated that low levels of

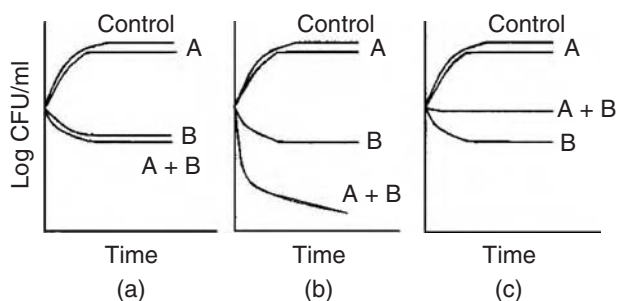


FIGURE 21.9 Isobologram permitting the interpretation of dilution results for combined antimicrobials testing. (a) Additive effect, (b) synergistic effect, (c) antagonistic effect.

ethylenediamine tetraacetic acid (EDTA) acted synergistically with nisin and lysozyme against *L. monocytogenes* but EDTA and monolaurin interacted additively against this microorganism. EDTA also synergistically enhanced the activity of nisin, monolaurin, and lysozyme against two enterohemorrhagic *E. coli* strains.

Fractional lethal concentrations (FLCs) can be determined when a broth dilution assay is used. This is the concentration of single or combined agents that kills the population of test organism to a nonrecoverable level under the test conditions. The FLC is determined by inoculating broth media without antimicrobial agents with aliquots from those samples in the MIC test that inhibited growth. The concentrations that had a lethal effect remain negative for growth after appropriate incubation, but growth is observed in the concentrations that had only an inhibitory effect.

Inhibition or time-kill curves (log CFU/ml vs. time) can be graphically interpreted for combination interaction (Barry, 1976; Krogstad and Moellering, 1986; Eliopoulos and Moellering, 1991). Because this technique uses a fixed concentration of each antimicrobial and must be repeated when a concentration is altered, it is logistically difficult and is most often used to enhance results observed with dilution methods. Survivor curves are generated with four treatments: (1) compound A alone, (2) compound B alone, (3) compounds A and B together, and (4) control A without A or B (Figure 21.9). Additivity is observed when the time-kill curve for the combined antimicrobials is similar to the curve for the more active compound alone (Figure 21.9a). Antagonism results if the combined effect is significantly less than that observed for either compound alone (Figure 21.9c). If the time-kill curve for the combined compounds is significantly reduced from both individual curves, the result is interpreted as synergistic (Figure 21.9b).

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Index

A

- ABC-transporter, 394
Acetaldehyde, 51, 147
Acetates
 antimicrobial activity, 101–102
 applications, 103
 regulation, 103
 toxicology, 102
Acetic acid
 antimicrobial activity, 94–99
 applications, 101
 daily intake, 101
 hurdle approach, 98–99
 lysozyme inhibition, 367
 metabolism, 16
 and propionic acid, 121
 regulation, 101
 sprays, 553
 temperature and, 95
 toxicology, 99–100
Acetobacter, 94
 sorbates and, 55
 sulfur dioxide and, 148–149
Acetobacter aceti, 148–149
N-acetylglucosamine, 361–362
Achromobacter, 55, 575
Acid-anionic surfactants
 antimicrobial activity, 534
 mechanism of action, 534
 pH and, 534
 toxicity, 536
 water hardness and, 534
Acid-base equilibrium, microbial, 124
Acid-blanch-chelate (ABC) process, 108
Acidified sodium chlorite (ASC), 509, 516
 fresh produce sanitization, 561
 poultry processing, 556–557
 red meat sanitization, 553
Acidifying, 622
Acidolin, 406
Acidophilin, 406
Acid rinses, 96, 97
Acids
 inorganic, 124–125
 organic. *See* Organic acids
 strong, 124
 weak, 124
Acid steam distillation, benzoic acid, 37
Acid tolerance
 development of, 126–127
 lactic acid bacteria, 92
Acidulants, 93–94, 640
Acinetobacter, 27, 55
Acridines, 641
Acrolein, 51
Actagardine, 391
Actinomycetes, 27
Actinoplanes liguriae, 391
Active transport, 125
Adaptation
 stress, 649–651
 to organic acids, 126–127
Additives, food, 1, 7–8
Additivity, antimicrobial
 combinations, 673–674
Adenosine triphosphate (ATP), 125, 639
Adenosine triphosphate (ATP)-binding cassette transporter (Pdr12), 56
Adherence-blockade, 473, 475
Adhesins, 464
Adipic acid, 105–106
Adsorption, avidin purification, 480
Adzuki bean paste, 375
Aeromonas, 55
Aeromonas hydrophila
 lactic acid and, 114–115, 118
 parabens and, 293, 294–295
Aeromonas salmonicida, 468
Affinity chromatography
 avidin purification, 480
 lactotransferrin, 462
Aflatoxin production
 benzoic acid/benzoate and, 16, 17
 lactic acid and, 116
 lactoperoxidase and, 459
 natamycin and, 277
 nitrite and, 216
 organic acids and, 19
 polyphosphates and, 582
 propionic acid and, 122
 salt concentration and, 577
 sodium bicarbonate sprays, 588
 sorbic acid/sorbates and, 54–55, 65
Agar diffusion assays, 662, 663–664, 673
Agar dilution assays, 664–666, 673
Agar plates, bacteriocins assay, 397–398
Agglutination, 473, 475
 β -alanine, and sodium propionate, 122
Albumin
 egg, 362, 479
 serum, 330
Alcaligenes, 55
Alcaligenes eutrophus, 27
Alcaligenes visclactis, 293
Alcohol beverages
 nisin and, 256
 sulfite levels, 157
Alcohol dehydrogenase, 69, 522
Alcohols
 dimethyl/diethyl dicarbonate and, 321
 and lysozyme assays, 363
 mycobacteria and, 644
Alcohol and Tobacco Tax and Trade Bureau, 157
Aldehydes, in fruit juices, 146–147
Aldolase, 200
Alfalfa, 96
Alginate
 binder, 120
 lysozyme inhibition, 367
Alicyclobacillus acidoterrestris, 255
Alkylating agents, 641
Alkylbenzene sulfonate, 536
Alkyldimethylbenzylammonium chlorides, 529
Alkyl halide, 529–529
Alkylphenoxypolyglycol ethers, 522
Allergenicity
 egg-white lysozyme, 378
 lactotransferrin, 471
 sorbic acid, 72
Allicin, 439, 441
Allium, antimicrobial properties, 439–440
Allspice, 431–432
Allylaminothiocarbonyl sulfonate, 147
Allyl isothiocyanate, 9, 147
Aloe vera, 617
Alternaria, 54, 154
 lactolipids and, 484
 monolaurin and, 343
Alteromonas, 55
Alteromonas putrefaciens, 68, 69
American Cancer Society, nitrite safety, 219
Aminacrine, 641
Amino acid oxidases, 17

- Amino acids
 chlorine dioxide and, 511–512
 deprivation, 650
 diethyl dicarbonate and, 313
 leakage of, 637
- Amino acid uptake
 benzoate and, 16
 fatty acid inhibition of, 346–347
 parabens and, 297
- Amino butyric acid, 239
- Aminoglycosides
 residues in milk, 610, 612
 resistance development to, 646
- Ammonia, dimethyl/diethyl
 dicarbonate and, 320
- Ammonium bicarbonate, 588
- Amphotericin, 276
- Amphoteric surfactants, 536–537
- AMS (Automicrobic) system, 668
- Anaphylaxis, benzoic acid/benzoate-associated, 37
- Ancovenin, 391
- Angkak, 210
- Animal agriculture, use of
 antibiotics/antimicrobials, 599
- Animal Health Institute, 599
- Anionic surfactants, mechanism of action, 639
- Anions
 lactotransferrin binding, 463
 transport, 17
- Anise, 295, 435, 438
- Antagonism
 antimicrobial combinations, 674
 organic acids, 21–22
- Anthocyanins, 147, 436
- Antibiotic/antimicrobial residues,
 599–600
 in aquatic systems, 600, 615
 in eggs, 612–614
 in fish, 614–615
 incidence of, 601–609
 maximum residue level (MRL),
 600
 in milk, 609–612
 nonviolative, 604–606
 resistance development and, 600,
 615–616
- Antibiotics, 633
 in aquatic systems, 600, 615
 bacterial resistance, 648–649; *see also* Resistance
 development
 discovery of, 328
 lactotransferrin and, 465–466
 withdrawal times, 600
- Antibiotic susceptibility testing, 661;
see also Antimicrobial
 testing
- Antibodies. *See* Immunoglobulins
- Antibrowning compounds, 2
- Antigens
 diversity of, 472
 immunoglobulin binding, 478
- Anthelmintics, residues, 599
- Antimicrobial combinations, *see also*
 Hurdles; Hurdle
 technology
 with acetic acid, 98–99
 additivity, 673–674
 antagonistic, 674
 assays, 672–674
 with EDTA, 586–587
 with fatty acids, 348–352
 with lactates, 119–120
 with lysozyme, 371–375
 with monolaurin, 340342–346
 with nisin, 248
 with organic acids, 20–22, 32
 with polyphosphates, 583
 with propionic acid, 121–122
 sanitizers, 554
 with sorbic acid/sorbate, 59–60
 synergism, 673–674
- Antimicrobial residues. *See*
 Antibiotic/Antimicrobial
 residues
- Antimicrobials, *see also* Sanitizers
 activity, 5, 8, 660–661
 biofilms and, 647
 cost, 8
 defined, 2, 430
 effect on cytoplasm, 641
 effect on cytoplasmic membrane,
 637–641
 effect on outer cell layers,
 635–636
 efflux, 645–646
 fatty acids, 533–536
 FDA definition, 2
 food-related interactions, 5–6
 function of, 2
 future of, 9
 inactivation, 647–648
 indirect, 574–592
 labeling, 8
 mechanism of action, 634–642
 packaging and, 6–7
 potentiation by chelators, 6
 preservation process and, 6–7
 properties, 5
 resistance. *See* Resistance
 development
 selection of, 3–7
 sensory effects, 8
 spectrum, 4–5
 synergistic activity, 9
 target sites, 634–635
 toxicology, 7–8
 traditional, 2
 types of, 633
- use of, 7–9
- Antimicrobials, natural, 2, 634
 animal sources, 455
 avidin, 479–481
 defensins, 484–485
 immunoglobulins, 472–479
 lactolipids, 481–484
 lactoperoxidase, 456–460
 transferrins, 460–472
 heat and, 623
 labeling, 8
 plant sources, 429–432, 439–440
 activity, 441–442
 applications, 442–444
 efficacy of, 432–435
 mechanism of action,
 440–441
 phenolics, 435–439
- Antimicrobial testing
 agar diffusion, 663–664
 application studies, 674–675
 automated methods, 668–670
 combined systems, 672–674
 descriptive methods, 670–672
 dilution methods, 664–666
 gradient plates, 666–670
 interpretation, 675–677
in vitro methods, 663–666
 principles of, 660
 standardization, 661
 test organism, 660–661
 time-kill curves, 670–671
- Antioxidants, 2
 in aquatic systems, 615
 citric acid and, 110
 phenolic, 435–436, 443
 sorbates and, 59–60
- Antistaling compounds, 2
- Aplin & Barrett Ltd., 238, 240
- Apo-lactoferrins, 467
- Apple cider, 32
 dimethyl/diethyl dicarbonate and,
 316
 fermented, 146
 sorbic acid/sorbate and, 59, 64
 sulfur dioxide in, 149
- Apple juice, natamycin and, 283
- Apple purée, vanillin and, 444
- Apples
 coatings for, 33
 hydrogen peroxide sanitization,
 561
 moldy, 146
 peroxyacids sanitization, 561
 sulfur dioxide and, 154
- Application studies, antimicrobials,
 674–675
- Aquatic systems, residues in, 600, 615
- Arabinose, 146
- Arachidonic acid, 330
- Arcobacter*, nisin and, 244

- Arcobacter butzleri
 citric acid and, 107
 EDTA and, 585
 Arteriosclerosis, natural phenolics
 and, 445
Arthrobacter, 55
Arthrobacter pascens, 109
Arthrobacter simplex, 109
Ascochyta, 54
 Ascorbate
 EDTA and, 586
 iron sequestration, 187
 nitrite and, 188–189
 and nitrosamine inhibition, 199
 Ascorbic acid
 benzoate and, 29
 nitrite and, 186
 sulfites and, 155
Ascosphaera, 54
Ascosphaera aggregate, 68
 Ascospores, heat-resistant molds, 109
 Asian sauces, 64
 Aspartase, 69
Aspergillus, *see also* Aflatoxin
 production
 essential oils and, 438
 fruit spoilage, 154
 parabens and mycotoxin
 production, 296–297
 sorbates and, 54, 57
 vanillin and, 438, 442
Aspergillus clavatus, 122
Aspergillus flavus
 benzoic acid and, 33
 monolaurin and, 343
 natamycin and, 277, 284
 parabens and, 296
 propionic acid and, 122
 sodium diacetate and, 103
 sorbic acid and, 54–55
Aspergillus fumigatus, 103
Aspergillus glaucus, 103, 591
Aspergillus nidulans, 280
Aspergillus niger
 acetic acid and, 94, 99
 benzoate metabolism, 27
 chlorine and, 515
 fatty acids and, 330, 331
 lactolipids and, 484
 peroxyacetic acid and, 548
 propionic acid and, 122
 propylene glycol and, 591
 recombinant lysozyme, 378
 sorbates and, 64
 sulfur dioxide and, 154
Aspergillus ochraceus, 277, 284
Aspergillus oryzae, 319
Aspergillus parasiticus
 acetic acid and, 99
 aflatoxin production, nitrite and,
 216
 benzoate and, 17
 chlorine and, 515
 citric acid and, 109
 lactic acid and, 116
 monolaurin and, 343
 natamycin and, 277
 salt tolerance, 577
 sorbic acid and, 54–55
Aspergillus versicolor, 109
 Assays, antimicrobial. *See*
 Antimicrobial testing
 Asthma, sulfite hypersensitivity, 156
 ATPase, 298
 Autobac, 668
 Autosterilization, 624
 Avidin, 362, 479–481
Azotobacter vinelandii, 584
- ## B
- Bacillus*, 472
 acetic acid and, 94
 biocides and, 635
 in canned meat, 187
 lysozyme and, 368
 nitrite/nitrate and, 177
 resistance development, 643
 sodium lactate and, 116
 sorbates and, 55
Bacillus anthracis, 397, 661
Bacillus cereus
 citric acid and, 108
 EDTA and, 585
 fatty acids and, 335
 lactolipids and, 483
 in liver sausage, 204
 lysozyme and, 368, 369
 nisin and, 241, 252, 256
 organic acids sensitivity, 94, 95
 and potassium sorbate, 20
 pressure tolerance, 623
 salt tolerance, 576
 sodium diacetate and, 103
 sorbates and, 60, 66
Bacillus coagulans
 lactic acid and, 112
 lysozyme and, 368
 nisin sensitivity, 246
Bacillus fulva, 316
Bacillus licheniformis, 66
 monolaurin and, 342–343
 nisin and, 252, 254
Bacillus megaterium, 346–347
Bacillus mesentericus (subtilis), 103
Bacillus metiens, 517, 519
Bacillus polymyxa, 177
Bacillus pumilus, 252
Bacillus sporothermodurans, 241
Bacillus stearothermophilus, 252
 benzoate metabolism, 27
 lactic acid and, 114
 lysozyme and, 368, 379
 monolaurin and, 338
 nisin and, 246, 252, 257
 organic acids and, 20
 sorbates and, 59
Bacillus subtilis
 acid tolerance, 121
 benzoate and, 16
 ethanol and, 590
 eugenol and, 438
 iodophors and, 523
 lactolipids and, 483
 monolaurin and, 338
 propionic acid and, 120, 122
Bacillus thermosphacta
 and lactic acid salts, 118, 119
 nisin and, 253, 255
 nitrite and, 204
 Bacon
 nitrite/nitrate in, 172–173,
 189–190
 nitrosamine levels, 199
 sorbates and, 66
 Bacteria
 antibiotic resistance, 648–649
 avidin, 481
 biocide resistance, 648–649
 defensins and, 483, 485
 dimethyl/diethyl dicarbonate and,
 310
 hydrogen peroxide and, 539
 IgY and, 478–479
 lactoperoxidase and, 458–459
 lactotransferrin and, 463–466
 parabens and, 293–296
 peroxyacetic acid and, 544
 pH and, 92
 QACs and, 529
 salt tolerance, 575–577
 sorbate degradation, 57
 stress adaptation, 649–651
 sulfur dioxide/sulfites and,
 148–151
 Bacteriocins
 activity, 400–402
 assays, 397
 biosynthetic pathway, 392–393,
 395
 Carnobacterium, 410–411
 chemistry, 391
 classification, 239, 390–391
 gene clusters, 391–392
 lactobacilli, 406–410
 lactococci, 400–402
 Leuconstoc, 411–414
 mechanism of action, 396
 modeling, 399
 nisin, 237–261
 in packaging film, 414–415
 pediococci, 402–406
 plasmids, 401–402

- posttranslational modifications, 393–394
 - producer immunity, 395–396
 - production of, 398–399
 - protein engineering, 416
 - regulation, 415
 - resistance, 399–400
 - S50, 401
 - typing, 401
- Bacteriophage
 - acid-anionic surfactants and, 534
 - chlorine and, 514, 516
 - dimethyl/diethyl dicarbonate and, 310
 - hydrogen peroxide and, 542
 - iodophors and, 523
 - QACs and, 531
 - sodium hypochlorite and, 512
- Bacterium lactis aerogenes*, 649
- Bacteriuria, 37
- Bactin, 198
- Bactometer, 670
- Bakery products
 - benzoic acid/benzoate and, 29
 - leavening, 588
 - natamycin and, 284
 - propionic acid and, 122
 - sodium diacetate and, 103
 - sorbates and, 65
- Bananas
 - hurdle technology, 624
 - sorbates and, 60
 - vanillin and, 444
- Basil oil, 295
- Bayer AG, 306
- B-cells, 474
- Beef
 - corned, 180, 212
 - ground, 181
 - lactic acid and, 112–114, 118–120
 - lactoferricins and, 469
 - lactoperoxidase systems and, 460
 - lactotransferrin and, 470, 471
 - “microwave-ready,” 340
 - nitrite and, 181, 187
 - sequential carcass
 - decontamination, 625
 - sulfites and, 150
 - trim, 113
- Beer
 - dimethyl/diethyl dicarbonate and, 319
 - green, 152
- Benzaldehyde, 12
- Benzalkonium chloride, 640, 648
- Benzene, 28
- Benzenecarboxylic acid, 12
- Benzoic acid/benzoate, 11
 - adverse reactions, 37
 - antimicrobial activity, 17–20
 - applications, 29–34
 - assays, 37–38
 - chemistry, 12
 - and chitosan, 22
 - food constituents and, 28–29
 - fungicidal properties, 32–33
 - generation, 12
 - mammalian metabolism of, 34–37
 - mechanism of action, 12–17
 - microbial metabolism of, 26–28
 - and quinic acid, 22
 - regulation, 29
 - resistance development, 22–26
 - sorbic acid/sorbate and, 14–15, 33, 60
 - storage/handling of, 31–32, 34
 - synergistic/antagonistic effects, 20–22
 - temperature and, 31–32
 - toxicology, 34–37
- Benzoyl-coenzyme A, 27, 34
- Benzyl alcohol, 28
- Benzyl chloride, 528–529
- Berries, 154
- Best Foods, Inc., 49
- β -lactam antibiotics
 - residues in eggs, 614
 - residues in milk, 610–612
 - resistance development to, 646
- β -propiolactone, 641
- Beverages
 - benzene levels, 28
 - benzoic acid/benzoate and, 29
 - fruit, sorbates and, 64
 - nisin and, 255–256
- Bifidobacterium bifidum*, bacteriocins
 - of, 413
- Bifidobacterium bifidus*, 376
- Bifidobacterium breve*, 102
- Bifidocins, 413
- Biocides, 633
 - bacterial resistance, 648–649
 - concentration effects, 638
 - effect on cytoplasmic constituents, 641
 - effect on cytoplasmic membrane, 637–641
 - effect on outer cell layers, 635–636
 - resistance development, 642–647
 - stress adaptation and, 649–651
 - target sites, 634–635
- Biofilms
 - biocides and, 647
 - chlorine and, 513
 - lactoperoxidase systems and, 460
 - peroxyacetic acid and, 544
- Bioscreen, 668–669
- Biotene™, 460
- Biotin, and avidin, 479, 480
- BioTrace, 668
- Bisphenol, 638
- Bisulfite, 147, 148
- Blackberries, 12
- Black bread molds, 99
- Black spot, 30
- Black tiger shrimp, 614
- Blanching, 624
- Blood
 - lysozyme in, 362
 - in meat, 206
- Blue cheese, 62
- Bologna
 - and botulinum toxin, 180
 - nisin and, 254
 - nitrite and, 214
- Botulism
 - fish products and, 210–211
 - risk assessment, 195–198
 - toxin production, 295–296; *see also Clostridium botulinum*, toxin production
- Botrytis*, 54, 154
- Botrytis cinerea*
 - dimethyl/diethyl dicarbonate and, 310
 - sulfur dioxide and, 154
- Brandies, fruit, 256
- Bread
 - propionic acid and, 122, 123
 - sorbates and, 54, 65
- Breast tissue, lactotransferrin in, 461
- Brettanomyces*, 56
 - natamycin and, 284
 - sorbates and, 54
 - sulfur dioxide and, 152
- Brevicin, 399
- Brewer's yeast, 256
- Brimstone, 143
- Brine, 98
 - bacon curing, 172–173
- Brochothrix*
 - lysozyme and, 368
 - sodium lactate and, 116
- Brochothrix thermosphacta*, 66, 203
 - lactic acid and, 115
 - lysozyme-nisin and, 373
 - nisin and, 241
 - polyphosphates and, 582
 - soaps and, 329
- S-bromomethazine, 606
- Broth dilution assays, 664–666, 673
- Browning
 - nonenzymatic, 51, 72
 - sulfites and, 155
- Bulgarian, 407
- Burkholderia cepacia*, 298
- Butoxypolypropoxypolyethoxyethano
 - 1, 524, 527
- Butter
 - diacetyl and, 592
 - lysozyme and, 375

- microstructure, 627
 natamycin and, 282
 propionic acid and, 122
 salt in, 577
 sorbates and, 54
 wrappers, 103
n-butylamine, 321
 Butylated hydroxyanisole (BHA),
 59–60, 436
 lysozyme and, 367
 parabens and, 298
 Butylated hydroxytoluene (BHT),
 59–60, 298, 436
 Butyl paraben, 295–297
Byssochlamys, 154, 155, 283
Byssochlamys fulva, 310
Byssochlamys nivea, 21
 dimethyl/diethyl dicarbonate and,
 317
 salt tolerance, 577
- ## C
- Cabbage, 108
 Caffeic acid, 298, 436
 Cakes, sorbates and, 54
 Calcium, EDTA chelation, 585
 Calcium acetate, 101–103
 Calcium benzoate, 12, 29
 Calcium bisulfite, 157
 Calcium chloride, 574
 Calcium lactate, 116, 119
 Calcium propionate, 65, 121, 123, 367
 Calcium sorbate, 50–54, 63; *see also*
 Sorbic acid/sorbates
 Calcium sulfite, 157
 Calf Antibiotic and Sulfonamide Test
 (CAST), 602, 603
 Camembert, 62, 366, 370
Campylobacter
 lactic acid and, 114–115
 peroxyacetic acid and, 544
 polyphosphates and, 582
 sodium lactate and, 116
 sorbates and, 55
Campylobacter botulinum, 68
Campylobacter coli, 28
Campylobacter jejuni, 28
 acetic acid and, 98
 chlorine and, 512
 lysozyme and, 368
 organic acids and, 32
 salt tolerance, 576
Campylobacter sporogenes, 68
 Canadian Food Inspection Agency,
 614
 Cancer, natural phenolics and, 445
Candida, 472
 iodophors and, 523
 lactotransferrins and, 468
 salt tolerance, 577
 and sodium lactate, 117
 sorbates and, 54, 56
Candida albicans
 acid tolerance, 121
 benzoic acid and, 33
 lactolipids and, 484
 lysozyme and, 371
Candida parapsilosis, 513
Candida utilis, 484
 Capillary electrophoresis, sorbic acid,
 74
 Caprylic acid, 106
 Capsaicin, 438
 Caraway seeds, 438, 623
 Carbolic acid, 291
 Carbon dioxide
 and nisin, 258
 and sorbate, 59
 Carbon monoxide, 201
 β -carboxylacolein, 51
 Carcasses
 lactic acid rinses, 112
 multiple sequential hurdle
 preservation, 62
 nisin disinfection, 253–254
 organic acid treatment, 96–97
 polyphosphate washes, 582
 preevisceration washing, 625
 sanitizers, 552–559
 succinic acid sprays, 123
 Carcinogenicity
 ethyl carbamate, 320
 nitrite, 66
 nitrosamines, 66
 Carcinomas, lactotransferrin levels,
 461
 Cardamom, 438
 Carnobacteriocins, 391
Carnobacterium, 116, 390
 bacteriocins, 391, 410–411
 lysozyme-nisin and, 373
 nisin and, 255
 pediocins, 405
Carnobacterium divergens, 253
Carnobacterium piscicola, 329, 411
 Carnocins, 411
 Carrageenan, 120
 Carrots, 108, 469
 Carvacrol, 9, 437–438
 antimicrobial activity of, 432
 heat and, 623
 nisin and, 248
 pH and, 441–442
 Carvone, 248, 438, 623
 Casein, 443, 456–457
 Casings
 bacteriocins in, 415
 natamycin in, 283
 nisin uptake, 255
 Castor oil soap, 328
 Catalase, 69
 Catechins, 436
 Catechol 1,2-dioxygenase, 27
 Catfish, 30, 102
 Cations
 EDTA chelation, 585
 in foods, 674–675
 polyphosphates and, 580–581
 Cattle, FSIS residue survey, 603–609
 Celery, 438
 Cell membranes
 acid amines and, 534
 chlorine and, 511
 lactotransferrin and, 463–466
 QACs and, 529
 sorbates and, 69
 Cell metabolism, sorbate inhibition of,
 69
 Cell surface, antimicrobials and,
 635–636
 Cell walls
 lysozyme and, 364, 371–375
 mycobacteria, 644–645
 Celsius system SURE, 668
Cephalosporium, 54
 Cetrimide, 640
 Cetylpyridinium chloride, 559
Chaetomium, 54
 Challenge tests, botulinal, 196
 Chalmaugrate, 331
 Champagne, 65, 152
 Charcoal, 330
 Charm 4000, 668
 Checkerboard method, combined
 antimicrobial testing,
 673
 Cheese
 aging, 366
 bacteriocins and, 401
 benzoic acid in, 12
 blue, 62
 Camembert, 62, 366, 370
 cold pack, 112
 cottage, 54, 63, 282, 338–339, 401
 Edam, 370, 375
 Emmentaler, 375
 farmhouse, 238
 Gouda, 251, 370, 375
 Hispanic type, 31, 251
 histamine in, 413
 Italian, 282, 371
 late blowing, 249, 366, 370, 375
 Manchego, 413
 mold-ripened, 401
 monolaurin and, 339
 mozzarella, 62–63, 370
 natamycin and, 280–282
 nisin and, 249–251
 nitrite/nitrate and, 210
 paneer, 251
 pasteurized processed, 249–250
 propionic acid and, 122, 123

- provolone, 375
 queso fresco, 63, 251
 Ricotta, 251
 Roquefort, 62
 salt in, 577
 shredded, 282
 soft, 31, 63, 251
 and sorbic acid/sorbate, 54, 61–63
 spreads, 103
 starter cultures, nisin-production, 250–251
 Swiss, 123, 281, 538
 wraps, 106
- Chelating agents, 636
 antimicrobial potentiation, 6
 EDTA, 583–587
 gram-negative bacteria and, 6, 584, 636
 gram-positive bacteria and, 6, 636
 lysozyme and, 364, 369, 371–375
 mechanism of action, 636
 monolaurin and, 335
 nisin and, 245, 248
 polyphosphates and, 580–581
- Chemiosmotic theory, 639
- Chemotherapeutic drugs, 633
- Cherries, 64
- Chicken, 370
 botulinal challenge tests, 196
 dimethyl/diethyl dicarbonate and, 318
 monolaurin and, 342
 nitrite and, 205
 organic acids and, 32
 ovoglobulins, 476
 propyl paraben and, 295
 sodium diacetate powder, 104
- Chicozapote, 624
- Chiller water
 chlorine in, 556
 lactic acid in, 115
 ozonated, 559
 trisodium phosphate in, 558
- Chilling, 622
- Chilling injury, and sodium benzoate, 33
- Chitinases, 440
- Chitosans, 22, 623
- Chlamydia trachomatis*, 483
- Chloramine, 515
- Chloramphenicol, 648
 residues in eggs, 614
 residues in milk, 612
- Chlorhexidine, 645
- Chlorine/chlorine compounds, 509–511
 antimicrobial activity, 512–516
 free available chlorine, 509
 fresh produce sanitization, 560–561
 lysozyme inhibition, 367
 mechanism of action, 511–512
 organic matter and, 519–521
 pH and, 516–519
 poultry sanitization, 556–557
 red meat sanitization, 553
 resistance development, 512–516
 temperature and, 519
 toxicity, 521–522
 water hardness and, 521
- Chlorine dioxide, 511
 fresh produce sanitization, 560–561
 mechanism of action, 511–512
 organic matter and, 521
 pH and, 517
 red meat sanitization, 553
 sporicidal activity, 516
 toxicity, 522
- Chlorobenzenes, 556
- Chloroform, 521, 556
- Chlorophenols, 556
- Chlorous acid, 553
- Chlortetracycline, 606
- Cholesterol, 330, 483
- Cholesterol-free reduced-calorie mayonnaise (CFM), 95
- Chromatography
 affinity, 462, 480
 benzoic acid/benzoate, 37
 gas, 301, 321, 590
 gas-liquid, 37, 482
 high-performance liquid (HPLC), 74, 287, 482
 ion-exchange. *See* Ion-exchange chromatography
 sorbic acid, 74
 thin-layer, 300
- Chymotrypsin, 247, 458
- Cider. *See* Apple cider
- Cinnamic aldehyde, 432, 436, 437, 623
- Cinnamon, 431–432
 activity of, 436–437
 bark, 436–437
 oil, 437
 phenolic coefficient, 435
 and sorbic acid, 442
- Cinnamycin, 391
- Circular dichroism, 457
- Cirrhosis, natural phenolics and, 445
- Citral, 443
- Citrate, and monolaurin, 335
- Citric acid, 636
 antimicrobial activity, 107–110
 and antioxidants, 110
 applications, 110
 fatty acids and, 349
 regulation, 110
 sprays, 553
 toxicology, 110
- Citric acid cycle, 16
- Citrinin, 281
- Citrobacter freundii*, sulfites and, 150
- Citronelol, 439
- Citrus fruit, 515
- Cladosporium*, 54, 154, 484
 monolaurin and, 343
- Clopidol, residues in eggs, 614
- Clostridium*
 acetic acid and, 94
 biocides and, 635
 nisin and, 249–250
 PA3679, 246, 252
 resistance development, 643
 salt tolerance, 575
 sorbates and, 55
- Clostridium bifermentans*, 187
- Clostridium botulinum*
 acetic acid and, 98
 in cheese, 250
 and curing brines, 175
 lactolipids and, 483
 lysozyme and, 2, 368, 369, 371, 636
 nisin and, 2, 241, 248, 253
 nitrite/nitrate and, 2, 171, 201
 propylene glycol and, 591
 salt tolerance, 576
 sorbic acid/sorbates and, 58, 60, 66
 sulfites and, 150
 toxin production
 in bacon, 189–190
 and curing brine, 180–181
 EDTA and, 586
 fermented meat, 188–189
 fish products, 210–211
 fumaric acid and, 111
 garlic/onion and, 439
 monolaurin and, 342
 parabens and, 295–296
 perishable canned cured meat, 185–188
 polyphosphates and, 580–581
 risk assessment, 195–198
 and salt concentration, 181
 sodium lactate and, 117–119
 sodium propionate and, 121
 vacuum-packaged meat, 188
- Clostridium butyricum*, 249–250
- Clostridium pasteurianum*, 201
- Clostridium perfringens*
 in canned meat, 187
 in cured meat, 206
 fatty acids and, 481
 and lactic acid salts, 118
 lysozyme and, 368, 369, 636
 nitrite and, 201, 202
 parabens and, 293
 Perigo factor and, 185
 salt tolerance, 576
 sorbates and, 66

- Clostridium putrefaciens*, 171
Clostridium putrificum, 171
Clostridium sporogenes, 66
 acetic acid and, 98
 in canned meat, 187
 in cheese, 250
 citric acid and, 108
 EDTA and, 585
 monolaurin and, 339, 342
 nitrite and, 202
 polyphosphates and, 580
Clostridium subterminale, 182
Clostridium thermosaccharolyticum, 246
 lysozyme and, 368, 379
 nisin and, 252
Clostridium tyrobutyricum, 210
 in cheese, 249–250
 lysozyme and, 364, 366, 368, 370, 375
 polyphosphates and, 581
 Clove, 431–432, 434, 436, 437
 and potassium sorbate, 442
 Coatings
 benzoic acid in, 33
 lysozyme in, 375
 lysozyme-nisin in, 376
 natamycin in, 280
 nisin in, 253, 254–255
 parabens in, 299
 polyvinyl acetate, 280
 Cobalt, 367
 Coccidia
 chlorine and, 5151
 iodophors and, 523
 Coccidiostats
 in aquatic systems, 615
 residues in eggs, 614
 Code of Federal Regulations, 8, 75
 Codex Alimentarius Commission
 antibiotic residues, 614
 nisin regulations, 259
 Coffee, 436
 Colicins, 390
 Coliforms, lysozyme-EDTA and, 372
 Collagens, 464
Colletotrichum, 54
 Colony hybridization, bacteriocins
 assay, 398
 Colorimetry
 EDTA, 587
 sorbic acid, 73–74
 Colostrum
 bovine, 473–476
 complement system, 475–476
 human, 362
 lactoglobulins, 473–476
 lactoperoxidase in, 456
 porcine, 460, 461
 Competitive inhibition, in cured
 meats, 203
 Complement activation, 472–473
 milk/colostrum, 475–476
 Conalbumin, 378, 471
 Concentration exponent, 638
 Condiments, 101
 Confectionary products, 67
 Conserving, 622
 Constant region, immunoglobulins,
 474
 Cooling, 2
 COPACT, 37
 Copper, 28
 chelation, 584
 lysozyme inhibition, 367
 Coriander, 435, 438
 Corned beef, 180, 212
 Coronavirus, IgY and, 479
Corynebacterium, lactolipids and, 483
Corynebacterium bovis,
 lactotransferrin and, 461
 Cosmetics
 benzoic acid/benzoate in, 33
 sorbates and, 67
 Cottage cheese, 401
 monolaurin in, 338–339
 natamycin and, 282
 sorbates and, 54
 and sorbic acid, 63
 p-coumaric acid, 298, 436
 Cocksackievirus, 515
 Cranberry juice, 37
 C-reactive protein, 461
 Cream, nisin and, 252
 Cream of tartar, 124
 p-cresol, 436
 Cross-adaptation, 649
 Cross-linking agents, 641
 Cross resistance, and hurdle
 technology, 628
 Crotonaldehyde, 51
 Crumpets, 256
Cryptocaryon irritans, 468
Cryptococcus, 54
Cryptococcus neoformans, 371
Cryptosporidium parvum, 515, 523
 Crystal violet, 641
 Cucumbers, 64, 409
 Cumene peroxide, 537
 Cumin, 438
Cunninghamella, 54
 Curd, wash water, 282
 Curing
 bacon, 172–173
 color development, 208
 salts, 173, 177–179
 Curvasin A, 396
 Curvaticins, 402
Curvularia, 54
 Cyanosis, 218
 p-cymene, 437–438
 Cysteine
 iron sequestration, 187
 and lysozyme, 373
 Cystic fibrosis, 461
 CYTED-D Programme, 624
 Cytokines, lactotransferrin and, 466
 Cytolysin, 392
 Cytomegalovirus, 336, 468, 483
 Cytoplasm, antimicrobials and, 641
 Cytoplasmic membrane
 effects of antimicrobials on,
 637–641
 enzyme inhibition, 640
 lactoperoxidase system and,
 458–459
 nisin and, 244
 parabens and, 297
 Cytosine, 147
- ## D
- Dairy products
 benzoic acid in, 12
 diacetyl and, 592
 fatty acids in, 338–339
 fortification of, 455
 lactoperoxidase and, 459–460
 lipids in, 481–484
 lysozyme and, 375–376
 nisin and, 249–252
 sorbic acid/sorbate and, 61–63
 Danisco, 240, 252, 275
 Danish Meat Research Institute, 177
 Data analysis, antimicrobial testing,
 675–677
 Davidson, P.M., 628
Debaromyces
 salt tolerance, 577
 and sodium lactate, 117
 sorbates and, 54
Debaromyces hansenii, 297
 Decanoic acid, 534
 Decenal, heat and, 623
 Decoquinat, residues in eggs, 614
 Defensins, 484–485
 Dehydroacetic acid, 104–105
 Dehydroalanine, 239, 391
 Dehydroascorbic acid, 145
 Dehydrobutyrine, 239, 391
 Delicatessen foods, 67
 Delvolid®, 275
 Dengue viruses, 483
 2-deoxyglucose, nitrite and, 200
 Dermatitis, contact, 300
 Dermatomycoses, 330
 Detergents
 in aquatic systems, 615
 quaternary ammonium
 compounds, 528–533
 Dextran, -lysozyme, 376
 Diacetate, and *L. monocytogenes*, 2

- Diacetyl, 592
- Diacyl peroxides, 537
- Dialkyl dicarbonate, synthesis, 308
- Dialkyl quaternary compounds, 529
- Diamidines, gram-negative bacteria and, 645
- Dichlorobenzyl alcohol, 33
- Dichlorodimethylhydantoin, 519, 521
- Dichloroisocyanuric acid, 515–516
- Dicranin, 329
- Diethyl carbonate, assays, 321
- Diethyl dicarbonate (DEDC), 305; *see also* Dimethyl/diethyl dicarbonate
- Diethylene triamine pentaacetate, and lysozyme, 373
- Diethyl pyrocarbonate, 305
- Dill, 438
- Dilution assays, 662, 664–666
- Dilution coefficient, 638
- Dimethyl/diethyl dicarbonate
- antimicrobial activity, 309–310
 - assays, 320–322
 - beer and, 319
 - chemistry, 307–309
 - dips, 318
 - ethanol and, 312
 - in foods, 318
 - fruit juices and, 316–317
 - grape juice and, 317
 - mechanism of action, 313–314
 - pH and, 312
 - regulation, 306–307
 - soft drinks and, 318
 - temperature and, 310–312
 - toxicology, 319–320
 - wine and, 317
- Dinitrophenol, 347
- Dipicolinic acid, 635
- Diplococcin, 400
- Dips
- acidified sodium chlorite, 556–557
 - cetylpyridinium chloride, 559
 - citric acid, 108
 - dimethyl/diethyl dicarbonate, 318
 - EDTA in, 586
 - lactic acid, 112, 115
 - lysozyme in, 375, 376
 - polyphosphates, 582, 583
 - propylene glycol, 591
 - sodium bicarbonate, 588
 - teat, 338
- Disinfectants
- antimicrobial testing, 661–663
 - role of, 648
- Dithiothreitol (DTT), 377
- Divercins, 391
- Divergicins, 391, 392
- DNA
- cross-linking agents, 641
 - intercalating agents, 641
 - lactotransferrin and, 464
 - leakage of, 637
- Dodecylbenzene sulfonic acid, 533
- Dodecylidiphenyloxide disulfonic acid, 534
- Dot blots, bacteriocins assay, 398
- Drinking water
- chlorine in, 521
 - nitrite in, 219
- Drugs, chemotherapeutic, 633
- Drying, 3, 622
- Dry salting, 430
- Ducks, ovoglobulins, 476
- Duramycin, 391
- Dyes
- antibacterial, 641
 - food-coloring, 147
- ## E
- Echinacea, 617
- Echoviruses, 515
- Edam, 370, 375
- EDTA, 583
- activity, 584–585
 - antimicrobial potentiation, 6
 - applications, 586–587
 - ascorbate and, 586
 - assays, 587
 - chemistry, 584
 - fatty acids and, 349–350
 - iron sequestration, 187
 - lactotransferrin and, 469
 - lysozyme and, 364, 369, 371–375, 584, 585, 586–587
 - mechanism of action, 585, 636
 - and monolaurin, 335
 - nisin and, 245, 587
 - nitrite and, 204, 585, 586
 - pH and, 636
 - QACs and, 531
 - regulation, 587
 - sorbates and, 51, 584
 - toxicology, 587
- Edwardsiella tarda*, IgY and, 478–479
- Effervescence, 305
- Efflux, antimicrobials, 645–646, 647
- Eggs
- antimicrobials in, 455
 - cured, 577
 - lysozyme in, 362
 - nisin and, 256
 - ovoglobulins, 476–479
 - residues in, 612–614
- Egg whites
- allergenicity, 378
 - avidin in, 479–481
- Electron transport system, 639
- ATP generation, 125
 - inhibition of, 640
 - and organic acids, 16
 - paraben inhibition of, 297
 - sorbate inhibition of, 69
- Electroporation, 248, 623–624
- Ellagic acid, 436
- Emetine, 308
- Emmentaler, 375
- Emphysema, natural phenolics and, 445
- Emulsifiers, nisin and, 248
- Endomycopsis*, sorbates and, 54
- Endotoxemia, lactotransferrin levels, 461
- Endpoint methods, antimicrobial testing, 670–672
- Enolase, 69
- Enterobacter*, 55, 213, 214
- Enterobacter aerogenes*, 104, 298
- Enterobacter agglomerans*, 150
- Enterobacter cloacae*, 118
- Enterobacter gergoviae*, 298
- Enterobacteriaceae
- acetic acid and, 98
 - benzoic acid metabolism, 26
 - lactic acid sprays, 112–113
 - nitrite/nitrate and, 204, 209
- Enterocins, 391, 412–413, 415
- Enterococcus*, 390
- bacteriocins, 391, 412–413, 415
 - in canned meat, 187
 - salt tolerance, 575
- Enterococcus faecalis*, 103
- Enterococcus faecium*
- bacteriocins, 412–413
 - EDTA and, 636
- Enteropathogens, nitrite/nitrate and, 212–215
- Enterotoxin production
- nitrite and, 191–192
 - polyphosphates and, 580–581, 582
 - salt concentration and, 576
- Environmental Protection Agency (EPA), chlorine in drinking water, 521
- Enzyme inhibition
- by benzoic acid/benzoate, 16–17
 - by dimethyl/diethyl dicarbonate, 313–314
 - by sorbate, 69
- Enzyme-linked immunosorbent assay (ELISA), nisin, 241
- Enzymes, cytoplasmic membrane, 640
- Eosinophil peroxidases, 457
- Epicidin, 280, 394
- Epidermin, 391, 392, 394
- Equipment, sterilization with
- hydrogen peroxide, 538, 539
- Ergosterol, polyene macrolides and, 280

- Erythorbate, 189
 and gluconolactone, 204
 and nitrosamine inhibition, 199
- Erythromycin, 465, 606, 612
- Escherichia coli*
 acid tolerance, 115, 121
 benzoate and, 16
 biocide susceptibility, 645
 caprylic acid and, 106
 citric acid and, 110
 EDTA and, 584, 636
 fatty acids resistance, 346
 hemorrhagic, 103
 IgY and, 478–479
 lactolipids and, 483
 lactotransferrin and, 464, 470
 monolaurin and, 338
 nisin and, 256
 nitrite resistance, 214–215
 organic acids and, 16, 25–26, 31–32, 95
 ovotransferrin and, 472
 parabens and, 297, 298
 polyphosphates and, 582
 QACs and, 529, 532
 resistance development, 646
 soaps and, 329
 sorbates and, 66
 SOS response, 650
 stress adaptations, 650
 sulfur dioxide and, 150
 verocytotoxigenic, 20, 60; *see also*
E. coli O157:H7
- Escherichia coli* K-12, 121
- Escherichia coli* O157:H7
 acetic acid and, 96, 113–114
 acid tolerance, 25
 in apple cider, 32
 cross-resistance, 648
 DHA and, 105
 diacetyl and, 592
 dimethyl/diethyl dicarbonate and,
 316
 fumaric acid and, 111
 growth-no growth interface,
 626–627
 and lactic acid salts, 117–119
 lysozyme and, 368, 370
 lysozyme-nisin and, 373
 nisin and, 253
 nitrite and, 215
 organic acids and, 25, 26, 95
 salt tolerance, 576
 sorbates and, 55, 57–58, 59, 64
 sucrose monolaurate and, 336
- Essential oils, 431–432
 antimicrobial activity of, 432–435,
 438
 applications, 442–444
 extraction methods, 432–435
 nisin and, 248
 plant sources, 440–441
 and salt, 442
 and yeasts, 440
- Ethanol, 589–590
 and benzoate, 32
 dimethyl/diethyl dicarbonate and,
 312
 lysozyme and, 367
 mechanism of action, 639
 and monoglycerides, 346
 oxidation by *Acetobacter*, 149
- Ethopabate, 614
- Ethyl acetate, essential oils extraction,
 435
- Ethyl carbamate, 306, 307, 320
- Ethylenediaminetetraacetic acid. *See*
 EDTA
- Ethylene oxide, 641
- Ethyl methyl carbamate, 321
- Ethyl paraben, 285–297
- 3-ethylphenol, 436
- Ethyl sorbate, 51
- Ethyl urethane, 317
- Eugenol, 343, 432, 436, 437, 438
- European Union (EU)
 lysozyme regulation, 379
 milk residues regulation, 612
 natamycin regulation, 281, 282
 nisin regulations, 259
 parabens regulation, 298
 sulfites regulation, 157
- European Union Project, hurdles, 621
- Eurotium amstelodami*, 122
- Eurotium herariorum*, 122
- Eurotium herbariorum*, 59
- Eurotium repens*, 122
- Eurotium rubrum*, 122
- Exocrine glands, lactoperoxidase
 synthesis, 456
- Exports, residue inspections, 601–602
- Extraction methods, sorbic acid,
 73–74
- ## F
- Fast Antimicrobial Screen Test
 (FAST), 602, 603
- Fat, nisin and, 246
- Fatty acids
 α -hydroxy, 333
 antibacterial, 328–330
 antifungal, 330–331
 applications in food, 484
 biosynthesis, 482
 cell membranes and, 534
 chemical modifications, 331–332
 chemistry, 482–483
 2-fluoro, 330
 history, 327
 hurdle technology, 348–352
 isolation/purification, 482
 lactolipids, 481–484
 lysozyme and, 363, 367
 mechanism of action, 346–348,
 483
 MICs, 332
 monoesters, 333–336
 monounsaturated, 482
 nisin and, 248
 polyunsaturated, 482
 resistance development, 346
 as sanitizers, 533–536
 saturated, 482
 specificity, 483
 stability, 483
 structure-function activity,
 332–333
 transport inhibition, 125
 unsaturated, 329–330
- Federal Register*, 551
- Feed, animal, 67
- Feline immunodeficiency virus, 468
- Fennel, 295, 435, 438
- Fentichlor, 347, 348
- Fermentation, 2, 589, 622
- Fermented products, lactic acid
 bacteria and, 94
- Ferredoxin
 clostridial, 187
 nitrite and, 201, 202
- Ferric chloride, 201
- Ferrous sulfate, 469
- Ferrylperoxidase, 458
- Ferulic acid, 298, 436
- Fibronectin, 464
- Ficin, 69
- Films
 bacteriocins in, 414–415
 EDTA in, 587
 lysozyme-nisin in, 376
 parabens in, 299
- Fimbriae, 464
- Fish
 antibiotic/antimicrobial residues,
 614–615
 botulism and, 196, 210–211
 curing, 210–212
 EDTA and, 586
 lactotransferrin and, 468
 monolaurin and, 342, 343
 nisin and, 255
 nitrite/nitrate and, 173, 196,
 210–212
 organic acids and, 30
 salt in, 577
 smoked, 196
 sorbates and, 54
- Flavobacterium*, 575
- Flavonoids, 436
- Flavonols, 436
- Flavons, 436
- Flavoring adjuvants, 106

- Flavoring extracts, 590
- Flavors, 8
- Fleming, Alexander, 328, 361, 662
- Flip-spot method, 664
- Flip-streak method, 664
- Flour-based products, and sorbic acid, 51
- Flow cell, bacteriocins assay, 398
- Fluoroquinolones, 614, 615
- Food
- acidity of, 92, 95, 107–108
 - defined, 600
 - dimethyl/diethyl dicarbonate and, 318
 - microstructure, 627
 - nisin and, 252–253
 - NOCs in, 218–219
 - novel, 622, 623
 - residues in, 599–600
 - sulfites in, 157
 - surface sanitizers, 552–562
 - target, 661
 - texture of, 8
 - total quality of, 622
- Food additives, 1, 7–8
- Food and Agriculture Organization/World Health Organization (FAO/WHO)
- chemicals in food, 430
 - dimethyl/diethyl dicarbonate regulation, 320
 - nisin regulation, 259
 - parabens regulations, 298, 300
- Food antimicrobials. *See* Antimicrobials
- Food Chemicals Codex, 583
- Food contact substance, 306
- Food and Drug Administration (FDA)
- antimicrobial agents, 2, 430
 - bacteriocins regulation, 415
 - Center for Veterinary Medicine, 600
 - chemical preservatives, 2
 - diethyl dicarbonate ban, 306
 - lactotransferrin regulation, 461, 471
 - lysozyme regulation, 378, 379
 - milk sampling survey, 609–612
 - nisin regulation, 389–390
 - sanitizer regulations, 551
 - sulfite regulations, 157
- Food, Drug and Cosmetics Act, Food Additives Amendment, 430
- Food preservation technology, 429–430
- Food preservatives, Code of Federal Regulations, 75
- Food Safety Inspection Service (FSIS)
- residue survey, 600–609
 - rinse systems, 97
- Formaldehyde, 641, 643
- Formic acid, 51
- Fractional inhibitory concentration (FIC), 442, 676
- Fractional lethal concentrations (FLC), 677
- Fragrances, in aquatic systems, 615
- Frankfurters, 370
- acid/heat treatment, 114
 - botulinal challenge tests, 196
 - and lactic acid salts, 118
 - nitrites in, 187, 214
 - pediocins and, 405
 - vacuum-packaging, 188
- Free radicals, benzoate scavenging of, 17
- Freeze/thaw, 374
- Freezing, 622
- Friend virus, 468
- Fructose, 146
- Fruit
- benzoic acid/benzoate and, 29, 32–33
 - dried, 64
 - hurdle technology and, 624
 - raw, sanitizers, 559–562
 - sorbates and, 54, 64–65
 - spoilage molds, 154
 - vanillin and, 443–444
- Fruit juice
- benzoic acid in, 12
 - combination preservation methods, 21
 - dimethyl dicarbonate and, 306
 - dimethyl/diethyl dicarbonate and, 316–317
 - natamycin and, 283
 - nisin and, 255
 - organic acids and, 22–23, 31
 - sorbates and, 54, 64
 - spoilage, 19
 - sulfur dioxide and, 146–147, 152
- Fumarase, 69
- Fumaric acid, 111–112, 342
- Fumarylpyruvate, 27
- Fungi
- benzoic acid/benzoate and, 17
 - chlorine and, 515
 - defensins and, 485
 - halotolerant, 30
 - lactoferricins and, 468
 - lactolipids and, 484
 - lactoperoxidase and, 459
 - lactotransferrin and, 464, 466, 467–468
 - parabens and, 296–297
 - peroxyacetic acid and, 544
 - polyphosphates and, 582
 - QACs and, 531
- Fungicides, benzoic acid/benzoate as, 32–33
- Fura, 51
- Fusarium*
- benzoic acid and, 17
 - monolaurin and, 343
 - parabens and, 296–297
 - sorbates and, 54, 57
- Fusarium oxysporum*, 122, 310
- Fusarium proliferatum*, 296
- Fusarium verticillioides*, 296
- Futile cycle, 25

G

- Galactomannan, -lysozyme, 376
- Galactose, 146
- Gallic acid, 436
- Gallidermin, 391
- Gamma irradiation, lysozyme inactivation, 367
- Garlic, 439–440, 617
- antimicrobial testing, 662
 - nisin and, 248
- Gas chromatography
- diethyl carbonate, 321
 - ethanol, 590
 - parabens, 301
- Gas-liquid chromatography
- benzoic acid, 37
 - fatty acids analysis, 482
- Gels, lysozyme in, 376
- Genes
- nisin, 258
 - S. natalensis*, 277
- Gentamycin, 465, 606, 610, 612
- Gentisate, 27
- Geotrichum*, 54
- monolaurin and, 343
 - sorbate degradation, 57
- Geotrichum candidum*, 515, 577
- Geraniol, 439
- German Agricultural Association, 544
- German Veterinary Association, 544
- Giardia lamblia*, 467, 468
- chlorine and, 515
 - lactolipids and, 484
- Ginger, 444
- Gingerol, 444
- Gingeron, 438
- Glaucoma piriformis*, 329
- Glucadium*, 54
- Glomerella cingulata*, 154
- Gluconate, 331
- Gluconeogenesis, 348
- Gluconic acid, 636
- Gluconobacter oxydans*, 25, 56
- Gluconolactone, and erythorbate, 204
- Glucuronic acid, 36
- Glucose
- in fruit juices, 146–147

- nitrite and, 200
storage, 482
- Glutamate, and sorbic acid, 72
- Glutamic acid, bacterial uptake, 347
- Glutaraldehyde, 635, 642
- Glycerol monolaurate, 20, 335–346
- α -glycerol phosphate, parabens and, 297
- Glycine, and lysozyme, 374
- Glycoaminoglycans, 464
- Glycocalyx, 647
- Glycolytic pathway, 125
- Glycopeptides, resistance development to, 646
- Goats, FSIS residue survey, 603–609
- Goldenseal, 617
- Gooding, C.M., 49
- Good Manufacturing Practices (GMPs), 625
- Gouda, 251, 370, 375
- Government Accounting Office, 609
- Gradient plates, 666–667
- Grains, sorbates and, 54
- Gram-negative bacteria
acid-anionic surfacants, 534
allicin and, 439
antimicrobial target sites, 634–635
avidin binding, 481
bacteriocins and, 396
chlorine and, 512
EDTA and, 6, 584, 636
fresh produce and, 559
hexachlorophene and, 645
hydrogen peroxide and, 539
lactic acid and, 114, 116–117
lactolipids and, 483
lactoperoxidase and, 459
lactotransferrin and, 46–468
lysozyme and, 372
lysozyme-lactoferrin and, 374–375
multidrug resistance, 650–651
nisin resistance, 242, 244, 245
and ovotransferrin, 472
parabens and, 293–296
peroxyacetic acid and, 547–548
polyphosphates and, 580
QACs and, 529, 635, 645
resistance development, 645
- Gram-positive bacteria
acid-anionic surfacants, 534
allicin and, 439
antimicrobial target sites, 634–635
avidin binding, 481
colicins/bacteriocins of, 390, 396
EDTA and, 6, 636
and fatty acids, 329–330
hydrogen peroxide and, 539
lactic acid and, 114, 116–117
lactolipids and, 483
lactoperoxidase and, 458–459
- lactotransferrin and, 464, 466–468
- lysozyme and, 364, 368, 372
- lysozyme-nisin and, 374
- nisin sensitivity, 241–244
- and ovotransferrin, 472
- parabens and, 293–296
- peroxyacetic acid and, 547–548
- and phenolics, 436
- polyphosphates and, 580–582
- QACs and, 529, 635
- Granulocytes, lysozyme in, 362
- Grape juice
dimethyl/diethyl dicarbonate and, 317
- fermentation of, 149–150
- sulfites in, 149
- sulfur dioxide and, 148, 151
- Grapes
botyriized, 146
- sulfites in, 157
- sulfur dioxide and, 154
- Gravies, meat, 255
- “Green chemicals,” 430
- Green olive extracts, 436
- Growth, microbial
modeling, 671
- pH and, 92
- sorbate inhibition of, 68–71
- Growth–no growth interface, 626–627
- Growth promotants, residues, 599–600
- Glyceraldehyde-3-phosphate dehydrogenase, 456
- Gypsum, 144
- ## H
- Hafnia*, 213
- Hafnia alvei*, 150
- Halides, 458
- Halogens, 509–522
- Halophiles, 575–577
- Ham
botulinal challenge tests, 196
- canned
curing salts, 173
- shelf stability, 178
- soured, 171
- dry cured, 207–209
- Karst, 209
- low brine, 213
- Parma, 209
- pH and, 213
- raw, natamycin and, 283
- red pigments, 209
- San Daniele, 209
- Savoie, 209
- Hanging mercury drop electrode (HMDE), 74
- Hansen’s disease, 331
- Hansenula*
benzoic acid and, 20
- salt tolerance, 577
- sorbates and, 54
- Hansenula wingei*, 588–589
- Hazard Analysis Critical Control Point (HACCP), 601
- Heat
and curing salts, 173
- and organic acids, synergistic effects, 21
- pH and, 623
- preservation, 2, 622
- Heat resistance, and sorbic acid/sorbate, 58–59
- Heat-shock proteins, 649
- Hsp26, 56
- Heat shock response
organic acids and, 15
- and sorbic acid/sorbate, 58–59
- Heavy chain, immunoglobulins, 474, 477
- Helicobacter pylori*, 336
- Helminthosporium*, 54
- Helveticins, 391, 408
- Hematin
-dependent nitrite reduction, 208
- lactotransferrin and, 469
- Heme
curing with NO, 199–200
- in lactoperoxidase, 457
- Hemin, 177
- Hepatitis C virus, 468
- Herbs, as antimicrobials sources, 429–446
- Herpes simplex virus, 412, 468, 483
- Herring, cured, 210
- Heterolactics, 94, 149
- Heterosporium*, 54
- Hexachlorophane, 640
- Hexachlorophene, 645
- Hexametaphosphates, 578
- Hexokinase, 456
- Hexylresorcinol, 30
- High-performance liquid chromatography (HPLC)
fatty acids analysis, 482
- natamycin, 287
- sorbic acid, 74
- High temperature processing, 622
- High temperature/short time (HTST) pasteurization, 475
- Hippuric acid, 12, 34–36
- Hippuricase assay, 28
- Histamine, 151, 413
- Histidine, 313
- Histidine protein kinase, 395
- Holofugizone, 614
- Homeostasis
microbial, and hurdle technology, 624

- pH, 124
 population, 624
- Homolactics, 94, 149
- Horizontal agar diffusion test, nisin, 240–241
- Hormones, in aquatic systems, 615
- Horses, FSIS residue survey, 603–609
- Hot dogs, propyl paraben and, 295; *see also* Frankfurters
- Human immunodeficiency virus (HIV)
 fatty acids and, 336
 lactolipids and, 483
 lactoperoxidase and, 459
 lactotransferrins and, 468
- Human influenza virus, 468
- Humectants, 591
- Humicola*, 54
- Hurdles
 European Union Project, 621
 multiple sequential, 625
 sublethal, 628
- Hurdle technology, 93; *see also*
 Antimicrobial combinations
 applications, 621–625
 defined, 7, 621
 EDTA and, 586–587
 fatty acids, 348–352
 lactate combinations, 119–120
 lactotransferrin and, 469–470
 logically-based, 624–625
 microbe-inactivation techniques, 623–624
 microstructure, 627
 multitargeting, 628–629
 and novel foods, 622, 623
 organic acids, 126–127
 pitfalls, 627–628
 polyphosphates, 583
 predictive modeling, 626–627
 sanitizers, 554
- Hydrogen peroxide, 145, 456, 458, 537
 antimicrobial activity, 539
 concentration effects, 539–510
 fresh produce sanitization, 561
 mechanism of action, 538–539
 mycobacteria and, 644
 organic acids and, 554
 pH and, 540
 poultry sanitization, 557
 reaction with pigments, 216
 red meat sanitization, 553–555
 sources, 538
 temperature and, 540–541
 tolerance to, 650
 toxicity, 542
- Hydrogen sulfide, 152
- Hydroperoxides, 537
- Hydroquinone, 436
- p-hydroxybenzoic acid, esters of, 291
- 3-hydroxybenzoyl-CoA, 27
- p-hydroxyhippuric acid, 300
- Hydroxysulfonic acids, 146
- Hy-Lite, 668
- Hypersensitivity
 benzoic acid/benzoate, 37
 sulfites, 156
- Hypochlorite, 509
 organic matter and, 521
 sporicidal activity, 517
 temperature and, 519
- Hypochlorous acid, 509–511, 556
- Hypothiocyanate, 458
- Hypothiocyanous acid, 458
- I**
- Ice cream, nisin and, 252
- Ichthyophthirius multifiliis*, 468
- Imidazoles, 313
- Immunity
 acquired, 455
 bacteriocins and, 396–396
 defensins and, 484–485
 lysozyme and, 362
 and nitric oxide production, 220–221
 passive, 455, 473
- Immunoassay, natamycin, 287
- Immunoglobulins
 biosynthesis, 474
 classes, 473
 functions of, 472–473
 IgA, 473, 477
 IgD, 473, 474
 IgE, 473
 IgG, 473, 477
 IgM, 473, 477
 IgY, 477–479
 isolation/purification, 474
 lactoglobulins, 473–476
 lactotransferrin and, 466
 ovoglobulins, 476–479
 stability, 475
 structure, 473–474
- Immunoregulation, lactotransferrin and, 465–466
- Impedance-splitting method, benzoic and sorbic acids, 37–38
- Impedance changes assays, 670
- Impermeability, microbial cell, 642–643
- Imports, residue inspections, 601–602
- Indifference, antimicrobial combinations, 673
- Inducible nitric oxide synthase (iNOS), 220
- Infant formulas
 acetic acid and, 101
 lactotransferrin and, 468
- lysozyme and, 376
- natural antimicrobials in, 455
- Infections
 lactotransferrin and, 466
 lysozyme and, 362
 and nitric oxide production, 220–221
- Infectious bursal disease, IgY and, 479
- Infinite inhibitory concentration (IIC), 671–672
- Inflammation
 and avidin production, 481
 lactotransferrin levels, 461, 466
 lysozyme and, 362
- Influenza virus, 483
- Inhibition zones, interpretation, 675
- Inoculum, antimicrobial testing and, 661
- Insecticides, in aquatic systems, 615
- Intercalating agents, 641
- Interleukins, lactotransferrin and, 466
- Intermediate moisture food (IMF) products, 622
- International Unit, nisin activity, 240
- Intestinal tract, lactotransferrin in, 460, 461
- Intrinsic factor effect profile, bacteriocins, 399
- Iodine, 522–523
 and lysozyme assays, 363
 mechanism of action, 641
 titration, 159
 toxicity, 528
- Iodophors
 antimicrobial activity, 523–524
 mechanism of action, 522–523, 642
 organic matter and, 528
 pH and, 524–527
 temperature and, 527–528
 water hardness and, 528
- Ion-exchange chromatography immunoglobulins, 474
 lactoperoxidase purification, 456–457
 lactotransferrin, 462
 sorbic acid, 74
- Iron, 28
 bacterial uptake, 464
 EDTA chelation, 584, 585
 in lactoperoxidase, 457
 lactotransferrin and, 461
 lactotransferrin binding, 463
 nitrite and, 183, 207
 ovotransferrin binding, 471–472
 sequestering, 187
- Irradiation
 lysozyme inactivation, 367
 shelf-stable canned cured meats, 177

- stress due to, 649
- Isoascorbate
- iron sequestration, 187
 - nitrite and, 198
 - and nitrosamine inhibition, 199
- Isobolograms, 675–676–7
- Isoborneol, 438
- Isobutylamine, 321
- Isoeugenol, 343
- Isoflavonoids, 440
- Isopropanol, mechanism of action, 639
- Isothiazolones, 640
- Italian cheeses, 375
- J**
- Jams, sorbates and, 64
- Japanese B encephalitis virus, 483
- Jellies, sorbates and, 64
- Jenseniin G, 414
- Jensen, L.B., 175
- Joubert, J.F., 397
- K**
- Kamaboko, 343
- Karst ham, 209
- Kefir, 401
- Kekessy-Piguet assay, 397–398
- β -ketoadipate pathway, 26–27
- α -ketoglutarate, 16, 147
- α -ketoglutarate dehydrogenase, 69
- Ketones, in fruit juices, 146–147
- Kheer, 252
- Khoa, 154
- Kimchi, 375
- Kinnow, 152
- Klebsiella*, 55
- lactotransferrin and, 470
 - nitrite and, 213
 - ovotransferrin and, 472
- Kloeckera*, 54
- Kloeckera apiculata*, 151, 438
- Kluveromyces marxianus*, lactolipids and, 484
- Kluveromyces lactis*, recombinant lysozyme, 378
- Koch, Robert, 661
- Kofi, 319
- Kojic acid, 30
- L**
- Labaneh, 63
- Labeling, antimicrobials, 8
- Lachrymal glands, 460
- Lactacins, 403, 407–408, 414
- Lactamase, 348
- Lactates, 116–120
- applications, 120
 - and *L. monocytogenes*, 2
 - and Microgard™, 119
 - and nisaplin, 119
 - and pediocin, 119
 - regulation, 120
- Lactic acid, 534
- antimicrobial activity, 112–116
 - applications, 116
 - dips, 112, 115
 - fatty acids and, 349
 - isomers of, 116
 - lysozyme inhibition, 367
 - regulation, 116
 - sprays, 112–114, 119, 553
 - toxicology, 116
- Lactic acid bacteria
- acetic acid production, 94
 - acid tolerance, 92
 - diacetyl and, 592
 - food-associated, 390
 - of fruits/beverages, 149
 - lysozyme and, 371
 - lysozyme-nisin and, 373
 - nisin and, 241, 256
 - nitrite and, 191, 203–204, 208
 - QACs and, 531
 - salt tolerance, 575
 - sodium lactate and, 116
 - sorbate degradation, 57
- Lactacins, 391, 392, 401, 402, 415
- in films, 414
- Lactobacillus*, 149
- acetic acid and, 97
 - bacteriocins, 406–410
 - diacetyl production, 592
 - green discoloration and, 216
 - lysozyme and, 368
 - pediocins, 405
 - sorbates and, 55, 66
- Lactobacillus acidophilus*, 149, 396, 401
- bacteriocins of, 406–411
 - lactocidin, 406
- Lactobacillus alimentarius*, 119
- Lactobacillus arabinosus*, 149
- Lactobacillus brevis*, 149
- Lactobacillus buchneri*, 149, 413
- Lactobacillus bulgaricus*, 401, 406–407
- Lactobacillus casei*, 109, 149, 310, 329
- Lactobacillus coryneformis*, 116
- Lactobacillus curvatus*, 329
- bacteriocins, 410
 - lysozyme-nisin and, 373
- Lactobacillus delbrueckii*, 149
- Lactobacillus delbrueckii* ssp. *lactis*, 371
- Lactobacillus delbrueckii* var. *bulgaricus*, 63
- Lactobacillus divergens*, 410–411
- Lactobacillus fermentans*, 103
- Lactobacillus fermenti*, 408
- Lactobacillus fermentum*, 149
- Lactobacillus helveticus*, 396, 408
- Lactobacillus hilgardii*, 149
- Lactobacillus innocua*, 410
- Lactobacillus lactis*, 203, 391
- Lactobacillus leichmannii*, 149
- Lactobacillus mesenteroides*, 373, 408–409
- Lactobacillus pentosus*, nitrite reduction, 208
- Lactobacillus piscicola*, 410–411
- Lactobacillus plantarum*, 94, 149, 402–403
- bacteriocins of, 408–409
 - Bactin and, 198
 - benzoic acid in, 12
 - citric acid and, 110
 - DHA and, 104
 - dimethyl/diethyl dicarbonate and, 312
 - nitrite reduction, 203, 204, 208
 - sodium propionate and, 121
- Lactobacillus reuteri*, 407
- Lactobacillus sake*, 329, 373, 391, 409–410
- nitrite reduction, 204
- Lactobacillus trichodes*, 149
- Lactobacillus viridescens*, 216, 254
- nitrite reduction, 203
- Lactocidin, 406
- Lactocins, 391, 392, 396, 408, 409–410
- Lactococci, 391, 392, 400–402, 403
- Lactococcus*, 116, 390
- bacteriocins, 400–402
 - soaps and, 329
- Lactococcus lactis*, 208
- nisin production, 258
 - soaps and, 329
- Lactococcus lactis* ssp. *cremoris*, 102, 251
- diplococci production, 400–402
- Lactococcus lactis* ssp. *diacetylactis*, 400–402
- Lactococcus lactis* ssp. *lactis*
- lactostreptins, 400–402
 - nisin production, 238, 389
- Lactoferricins, 460, 463, 465, 467–468
- Lactoferrin, 198, 460–461
- activated, 460–461, 462, 466, 470
 - and lysozyme, 374
- Lactoglobulins, 473–476
- applications, 476
 - biosynthesis, 474
 - chemistry, 473–474

- isolation/purification, 474
 mechanism of action, 475–476
 sources, 473
 specificity, 476
 stability, 475
- Lactolipids, 481–484
- Lactoperoxidase
 applications, 459–460
 biosynthesis, 456
 chemistry, 457
 mechanism of action, 458
 nisin and, 248, 459–460
 sources, 456
 specificity, 458–459
 stability, 457–458
- Lactoperoxidase-thiocyanate-
 hydrogen peroxide
 interaction, 456–460
- Lactose, 146
- Lactosiderophilin, 460
- Lactostreptins, 396, 400–401
- Lactotransferrin, 460
 allergenicity, 471
 application methods, 468
 applications, 468–470
 biosynthesis, 46, 4601
 blocking, 464
 chemistry, 462–463
 interactions, 464, 465–466
 isolation/purification, 462
 mechanism of action, 463–468
 MIC/MBC, 465
 regulation, 461
 resistance development, 465
 safety, 470–471
 sources, 461
 specificity, 466–468
- LanB, 394
- LanC, 394
- LanFEG, 395
- LanI, 395
- LanP, 394
- Lanthionine, 239, 391
- Lantibiotics
 biosynthesis, 392–393, 395
 genes, 392
 immunity, 395–396
 prelantibiotics, 393–394
 type A, 391
 type B, 391
- Las 5, 396
- Laurate, 329
- Laurel, 434, 438
- Lauribic™, 341, 343
- Lauric acid, 333, 347
- Lauric fatty acids, 327
- Lauricidin®, 336, 338, 343
- Lauri-lac™, 342
- Lead poisoning, 587
- Lecithin, 330
- Lectin pathway, complement
 activation, 475
- Leeks, 439–440
- Lemon grass oil, 433
- Lettuce, 156
- Leucocins, 391, 403, 412
- Leuconocins, 398–399, 412
- Leuconstoc*, 149, 390
 bacteriocins, 411–414
 diacetyl production, 592
 green discoloration and, 216
 nitrite and, 207
 pediocins, 405
- Leuconstoc citrovorum*, 411–412
- Leuconstoc dextranicum*, 411
- Leuconstoc gracile*, 149
- Leuconstoc mesenteroides*, 94, 203, 412
- Leuconstoc mesenteroides* ssp.
dextranicum, 149, 403
- Leuconstoc oenos*, 256, 310
- Leuconstoc paramesenteroides*, 412
- Leukocytes
 defensins in, 484–485
 lysozyme in, 362
- Light chain, immunoglobulins, 474, 477
- Lightning, 668
- Lime, 435
- Lincomycin, 606
- Linoleate, 327
- Lipase, 16
- Lipid II, nisin and, 244
- Lipid A, 466
- Lipids
 antimicrobial, 5–6, 329
 benzoic acid binding, 28
 in foods, 674
 parabens and, 297–298
 skin, 481
- Lipopolysaccharide (LPS)
 affinity of lactotransferrin, 466
 EDTA and, 636
 inhibition of organic acids, 126
 lysozyme treatment, 364, 369, 371–375
 and resistance development, 645
- Listeria*
 in ice cream, 252
 peroxyacetic acid and, 544
- Listeria innocua*
 nisin and, 253, 256
 parabens and, 295
 sodium lactate and, 116, 117
- Listeria ivanovii*, 295, 405
- Listeria monocytogenes*
 acetic acid and, 95–99
 acid-anionic surfactants and, 534
 acid tolerance, 25
 adaptive response, 127
 bacteriocin-resistant, 399–400
- benzoic acid/benzoate and, 16, 19–20, 28
- chlorine and, 512
- citric acid and, 107–108
- diacetate and, 2
- diacetyl and, 592
- essential oils and, 440
- fumaric acid and, 111
- growth-no growth interface, 627
- iodine and, 527
- lactic acid/lactate and, 2, 114–115
- lactolipids and, 483
- lactoperoxidase and, 459–460
- lactotransferrin and, 470
- lysozyme and, 368, 370, 373
- lysozyme-nisin and, 373
- monolaurin and, 338–340, 342
- nisin and, 241, 251, 253–254, 390, 396
- nitrite and, 202–203, 206–207
- organic acids and, 32
- parabens and, 294–295, 298
- pediocins and, 403–406
- polyphosphates and, 582
- potassium sorbate and, 63
- pressure tolerance, 623
- salt tolerance, 576–577
- ScottA, 295
- in seafood, 255
- in smoked fish, 211–212
- sodium acetate and, 102
- sodium diacetate and, 103–104
- sodium lactate and, 116–120
- sorbic acid and, 55
- thermotolerance, 623
- toxin production, 121
- trisodium phosphate and, 558
- Listeria seeligeri*, 295
- Lister, Joseph, 291
- Litchi nuts, 154
- Liver, iron content, 187
- Liver paté, 207, 254
- Liver sausage
 botulinal challenge tests, 196
 nitrite and, 204
- Livetin, 477
- Lobster, 255
- Longan, 154
- Lucenocin, 276
- Lumac, 668
- Luncheon meat
 canned, 184–185
 monolaurin and, 342–343
 nitrite and, 212–213
- Lymphocytes, defensins in, 484–485
- Lysine
 bacterial uptake, 347
 and sorbic acid, 72
- Lysozyme
 allergenicity, 378
 antimicrobial activity, 368–369

- applications, 375–376
 assays, 363–364
 bovine milk, 364
 and *C. botulinum*, 2
 chemistry, 361–362
 conjugates, 376
 EDTA and, 364, 369, 371–375,
 584, 585, 586–587
 effect on spores, 369–370
 egg-white, 364
 families of, 362
 and food-related organisms,
 370–371
 freeze/thaw and, 374
 and glycine, 374
 heat stability, 366–367
 human milk, 364
 hurdle approach, 371–375
 inhibitors, 363, 367
 and lactoferrin, 374
 modified T4, 378
 nisin and, 248, 373
 nonenzymatic antimicrobial
 activity, 377
 and polyethylene glycol, 374
 polyphosphates and, 583
 potentiation of, 371–375
 properties of, 362–365
 pulsed electric field treatment, 367
 recombinant, 378
 regulation, 8, 378–379
 resistance development, 368–369
 salt and, 367
 sources, 361–362
 stability in foods, 366–367
 target sites, 636
 and trisodium phosphate, 374
- ## M
- Macrolides, 276–277, 280
 in aquatic systems, 615
 residues in eggs, 614
 residues in milk, 612
 Macrophages
 avidin secretion, 481
 defensins in, 484–485
 lysozyme in, 362
 Magnesium cations, EDTA and, 585,
 636
 Magnesium chloride, 574
 Magnesium sulfate, lactotransferrin
 and, 469
 Malate dehydrogenase, 69
 Maleylpyruvate, 27
 Malic acid, 120
 Malolactic fermentation, 149–151
 Malonaldehyde, 51
 Malonic acid, 51
 Malthus system, 670
 Malting, sorbic acid during, 67
- Maltose, 146
 Malt syrups, 103
 Mammary glands, lactoperoxidase
 synthesis, 456
 Manchego cheese, 413
 Mandarin oranges, 154
 Manganese, lysozyme inhibition, 367
 Mangoes, 109
 hurdle technology and, 624
 and spice extracts, 444
 sulfur dioxide and, 154
 Mannose, 146
 Margarine
 diet, 336, 338
 microstructure, 627
 salt concentration, 577
 sorbates and, 67
 Mass spectrometry
 bacteriocins assay, 398
 fatty acids analysis, 482
 Mastitis, 338, 372, 461
 Matrix-assisted laser
 desorption/ionization
 time-of-flight mass
 spectroscopy (MALDI-
 TOF-MS), 398
 Mayonnaise
 and acetic acid, 95
 homemade, 108
 sorbates and, 67
 Measles virus, 483
 Meat
 bacteriocin-producing pediococci
 and, 404–405
 botulinal challenge tests, 196
 cooked, nisin and, 254–255
 cured
 botulinal risk, 195–198
 canned, 175–176
 C. perfringens in, 206
 green discoloration, 216
 nitrite burn, 216
 nitrite/nitrate in, 169–221
 nitrosamines in, 182
 Perigo factor, 179, 192
 perishable, 179–182, 193
 perishable canned, 185–188
 pH of, 203
 phosphates and, 581
 salmonellosis, 215
 salt in, 577
 shelf-stable, 177–179,
 182–185
 spoilage of, 203–207
 thermal process, 177–199
 fermented, 188–189, 207–209
 FSIS residue survey, 603–609
 fumaric acid and, 111
 and lactic acid salts, 118–120
 lactolipids and, 484
 lysozyme and, 375
- monolaurin and, 342
 natamycin and, 282–283
 nisin, 253–255
 organic acid treatment, 96–97
 pigment, NO-cured, 199–200
 polyphosphate washes, 582
 processing, sanitizers, 552–559
 raw, nisin and, 253–254
 red, sanitizers, 553–556
 slurries, 185–188
 sodium acetate and, 102
 sodium diacetate and, 103–104
 and sorbic acid/sorbates, 54,
 65–67
 sulfites and, 150, 157
 vacuum-packaged, 188–189
 Melanosis, 30
 Melanotransferrins, 460
 Membrane-active agents, 637–641
 Membrane transport, paraben
 inhibition of, 297
 Mercaptides, 640
 Mercaptoacetic acids, 331
 Mercuric chloride, 661
 Mercury, 640
 lysozyme inhibition, 367
 plasmid-encoded resistance,
 646–647
 Mersacidin, 391, 392
 Mesenteroicins, 412
 Mesophiles, acid blanching and, 108
 Metabisulfite, 624
 Metals
 and hydrogen peroxide activity,
 541
 lactotransferrin binding, 463
 Methanol, essential oils extraction,
 435
 β-methylanthionine, 239
 Methyl paraben, 291–301
 fatty acids and, 349
 sorbic acid and, 68
 Methyl sorbate, 51
 Metmyoglobin, 209
 Mettwurst, 214
 MIC analytical system, 616
 Micellar electrokinetic capillary
 chromatography
 (MECC), sorbic acid, 74
 Microbiology, predictive, 626–627
Micrococcus
 acetic acid and, 95
 in bacon, 189
 benzoate metabolism, 27
 lactolipids and, 483
 lysozyme and, 368
 nitrate reduction, 208
 nitrite and, 191
 sorbates and, 55
Micrococcus luteus, 240–241,
 363–364

- Micrococcus lysodeikticus*, 346–347
 Micro-Coder, 668
 Microcycin, 465
 Microfiltration, lactotransferrin, 462
 MicroGARD™, 119, 413
Microsporium, 19
 Microtiter tray tests, 668
 Milk
 acidified, 116
 antimicrobials in, 455
 bovine
 immunoglobulins in, 473–476
 lactoperoxidase in, 456
 lactotransferrin in, 460, 461
 complement system, 475–476
 fatty acids in, 338–339, 481–484
 goat, 415
 human
 lactoperoxidase in, 456
 lactotransferrin in, 460, 461
 hyperimmune, 476
 lactoperoxidase and, 459–460
 lysozyme in, 362, 370, 375, 376
 pasteurized, nisin and, 251–252
 porcine, lactotransferrin in, 460
 production, hydrogen peroxide in, 538
 residues in, 609–612
 skim, fermented, 401
 soy, 257
 sterile, 538
 triglycerides, 483
 Miller, E., 49
 Mineral supplements, 578
 Minimum inhibitory concentration (MIC)
 dilution assays, 665–666
 interpretation, 675
 spiral plating, 668
 Mirin, 366
 Miso, 484
 Mitchell, Peter, 639
 Modeling
 growth kinetics, 671
 hurdle technology, 626–627
 Molds
 ascospores, 109
 black bread, 99
 blue-vein cheese, 329
 on cheese, 280–282
 cinnamon and, 436–437
 dimethyl/diethyl dicarbonate and, 310
 essential oils and, 438
 fresh produce and, 559
 fumaric acid and, 111
 hydrogen peroxide and, 539
 monolaurin and, 343
 natamycin and, 277–279
 nitrite and, 215–216
 parabens and, 296–297
 polyphosphates and, 582
 propionic acid and, 122
 QACs and, 529
 quinic acid and, 22
 resistance development, 645
 salt tolerance, 577
 sodium diacetate and, 103
 sorbates and, 54, 56, 57
 sulfur dioxide/ sulfites and, 154–155
 vanillin and, 438
Monascus purpureus, 210
 Monier-Williams procedure, 158–159
Monilia, 54
Monilia taxa, 154
 Monoacylglycerols, 484
 Monocaprin, 338
 Monochloramine, 516, 517
 Monocytes
 defensins in, 484–485
 lysozyme in, 362
 Monoglycerides, 483
 antimicrobial effects, 335–336
 as emulsifiers, 349
 and ethanol, 346
 nisin and, 248
 purity of, 338
 temperature effects, 342
 Monolaurin, 336–346
 antimicrobial activity, 335–336
 and fumaric acid, 342
 and nisin, 339, 343
 and sorbic acid, 340–342
 Monopotassium phosphate, 102
 Monoterpenes, 623
Moraxella, 55
 in bacon, 189
 nitrite and, 204
Moraxella catarrhalis, 464
 Morpholine, 320–321
 Mortadella, 283
 Mouse mammary tumor virus, 483
 Mozzarella, 62–63, 370
 MS-2 Microbiology System, 668
Mucor, 27, 54, 57
Mucor miehi, 63
Mucor pusillus, 103
Mucor racemosus, 310
 Mucosa
 lactotransferrin production, 461
 peroxyacetic acid and, 551
 Mucus, lysozyme in, 362
 Multidrug resistance (MDR), 650–651
 Multiple-barrier concept. *See* Hurdle technology
 Multitargeting, hurdle technology, 628–629
 Mung bean, 96
 Muramidase, 362, 377
 Mustard, 24, 147, 438
 Mutagenicity
 nitrosamines, 346
 sodium benzoate, 37
 sorbic acid/sorbate, 72–73
 Mycobacteria, 55
 antimicrobial target sites, 635
 resistance development, 644–645
Mycobacterium bovis, 549–550
Mycobacterium fortuitum, 513
Mycobacterium tuberculosis, 513
 lactic acid and, 112
 soaps and, 329
 Mycolic acids, 645
 Mycostatin, and natamycin, 282
 Mycotoxin production
 fumitremorgins, 19
 natamycin and, 277–279
 organic acids and, 19
 parabens and, 295–297
 polyphosphates and, 582
 sorbic acid/sorbates and, 54–55, 67
 Myeloperoxidase, 457
 Myoglobin, 201
 Myristate, 327, 329
 Myristic acid, 347
 Myristicin, 444
Myrothecium verrucaria, 330
- ## N
- NADH, parabens and, 297
 Naphthalene-sulfonic acid, 534
 Natal Province, 275
 Natamax™, 275
 Natamycin
 antimicrobial activity, 277–279
 application methods, 280–281
 applications, 280–286
 assays, 286–287
 bakery products and, 284
 cheese and, 280–282
 chemistry, 276–277
 fruit juice and, 283
 meat and, 282–283
 and mycostatin, 282
 and potassium sorbate, 281–282, 283
 regulation, 259, 284–285
 sources, 275
 toxicology, 286
 National Academy of Science (NAS), dietary nitrite/nitrate, 194–195
 National Center for Clinical Laboratory Standards (NCCLS), 662
 National Conference of Interstate Milk Shippers, 609

- National Drug Residue Milk Monitoring Program, 609–610
- National Residue Program (NRP), 601, 609
- N-chloro-compounds, 517, 556
- Necteria galligena*, 25
- Neisseria*, 244, 464
- Neisseria gonorrhoea*, 483
- Neomycin, 606, 612
- Neosartorya fischeri*, 19, 38
- citric acid and, 109
- fumaric acid and, 111
- heat resistance, 59
- organic acids and, 31
- Nerol, 439
- Nickel, 587
- Nicotinamide adenine dinucleotide, 147
- Nisaplin®, 119, 239, 240
- Nisin, 391
- antimicrobial activity, 240–248
- applications, 248–258
- assays, 240–241
- beverages and, 255–256
- biosynthesis, 258, 392–393
- canned foods and, 252–253
- and carbon dioxide, 258
- and *C. botulinum*, 2
- chemistry, 239–240
- dairy products and, 249–252
- and EDTA, 587
- effectiveness, 389
- electroporation and, 623–624
- enzyme degradation, 247–248
- fat and, 246
- and lactic acid, 114
- and lactoperoxidase, 459–460
- and liquid egg, 256
- and lysozyme, 373
- meat products, 253–255
- mechanism of action, 244–248, 396
- monolaurin and, 339, 343
- and organic acids, 98–99
- and polyphosphates, 583
- production of, 398–399
- properties, 400
- proteases of, 394
- regulation, 8, 238–239, 259–262, 389–390
- resistance development, 245–246, 399
- safety, 238
- seafood and, 255
- sensitivity, 238, 239, 241–244
- and sodium metabisulphite, 246
- sources, 238, 239, 389
- storage losses, 247
- and sucrose palmitate, 336
- synergistic compounds, 248
- temperature and, 247
- thermophiles and, 623
- and titanium dioxide, 246
- toxicity, 259
- well diffusion assay, 664
- Nisin A, 239, 258
- Nisinase, 247–248
- Nisin Z, 239, 258
- Nitric oxide, 199–200
- effects of, 220–221
- nitrogenase inhibition, 201
- Nitric oxide synthases, 220–221
- Nitrite burn, 216
- Nitrite/nitrate
- alternatives, 199–200, 210
- ascorbic acid/ascorbate and, 175, 186, 188–189
- assays, 217–218
- in bacon, 172–173, 189–190
- bacterial reduction of, 207–209
- carcinogenicity, 66
- cheese and, 210
- clostridia and, 2, 172
- and cured meat spoilage, 203–207
- dietary, 219
- in drinking water, 219
- dry cured hams, 207–209
- EDTA and, 204, 585, 586
- enteropathogens and, 212–215
- in fermented meat, 188–189, 207–209
- fish and, 173
- health effects, 194–195
- inhibition, 193, 200–202
- iron and, 183
- L. monocytogenes* and, 206–207
- and meat slurries, 185–188
- molds and, 215–216
- Perigo factor, 182–185, 198–199
- perishable cured meat, 179–182, 185–188, 193
- regulation, 217
- resistance, 213–215
- S. aureus* and, 191–192
- seafood and, 210–212
- shelf-stable cured meats, 177–179
- and sodium isoascorbate, 186–187
- sorbate and, 72, 190
- and sulfites, 150
- use in foods, 170–171
- in vacuum-packaged meat, 188–189
- yeasts and, 215–216
- Nitrite reductases, 203–204, 214
- S-nitrocysteine, 200–201
- Nitrogenase, inhibition, 201
- Nitromide, 614
- Nitroprusside, 202
- Nitrosamines
- in bacon, 199
- carcinogenicity, 66
- in cured meats, 182
- exposure to, 219
- in foods, 170–171
- inhibition by benzoate, 17
- mutagenesis, 346
- Nitrosodimethylamine, 219
- Nitrosomyoglobin, 209
- Nitrous acid, 171, 177
- N-nitroso compounds (NOCs), 194–195, 199, 218–219
- Nocardia asteroides*, 28
- Nonanoic acid, 534
- Norwalk virus, 516
- Novobiocin, 612
- Nutmeg, 434, 444
- Nutraceuticals, resistance development and, 617
- Nystatin, 276, 282

O

- Ochratoxin, 277
- 9-octadecanoic acid, 533
- 1-octane sulfonic acid, 534
- Octanoic acid, 534
- Odors, 5, 8
- geranium-like, 57
- Oenococcus*, 149, 390
- Oenococcus oeni*, sulfur dioxide and, 149–150
- Off-colors, 5
- Off-flavors, 5
- Off-odors, 5
- geranium-like, 57
- Okra, 107
- Oleate, 327
- Oleic acid, 533
- Oleuropein, 436, 443
- Olives
- natamycin and, 277, 284
- phenolics from, 436
- sorbates and, 64
- Onion, 439–440
- Opsonization, 472, 475
- Optical density assays, 668
- Oral care products
- benzoic acid/benzoate in, 33
- lactoperoxidase systems, 460
- Orange juice, 283
- Organic acids, 436, 437–439
- Organic acids
- adaptation/resistance development, 126–127
- antioxidant/prooxidant properties, 36
- assays, 127
- combinations, 32
- effective use, 93–94
- and hydrogen peroxide, 554
- lipophilic, 16

- mechanism of action, 124–126, 639–640
- modeling inhibitory effects, 15–17
- and mycotoxin production, 19
- nisin and, 98–99, 248
- pH and, 640
- pKa, 93
- poultry sanitization, 557
- resistance development, 22–26
- Organic anthraquinone autooxidation, 538
- Organic matter
- chlorine/chlorine compounds and, 519–521
 - iodophors and, 528
 - peroxyacetic acid and, 549–550
 - QACs and, 532
- Organisation Internationale du Vin (OIV), 379
- Organochlorines, 509
- Organomercurials, 647
- Origanum oil, 200
- Ornithine cycle, 307
- Orthophosphates, 578, 581
- Osmoprotectants, 577
- Osmotic shock, 649
- Ovalbumin, 378
- Overprocessing, 627–628
- Ovoglobulins, 476–477
- applications, 479
 - chemistry, 477–478
 - isolation/purification, 477
 - mechanism of action, 478
 - sources, 477
 - specificity, 478–479
 - stability, 478
- Ovomucoid, 378
- Ovotransferrin, 378, 460, 471–472
- Oxidative phosphorylation, 16, 347–348
- Oxidative stress
- bacterial response to, 649, 650
 - organic acids and, 36
- Oxidoreductases, 460
- Oxygen, removing, 622
- Oxygen uptake, fatty acid inhibition, 346, 347
- Oxytetracycline, 606, 614
- Ozone
- fresh produce sanitization, 561–562
 - poultry sanitization, 559
 - red meat sanitization, 556
- P**
- Packaging
- and antimicrobial activity, 6–7
 - aseptic, 538, 539
 - bacteriocins in, 414–415
 - environmentally friendly, 622
 - lysozyme in, 376
 - parabens in, 299
 - vacuum, 177, 179–180, 188, 191
- Palmitate, 327, 329
- Palmitoleic acid, 333, 335
- Pancreatin, 247
- Pancreatitis, 461
- Paneer, 251
- Paneth cells, 484–485
- Papaya, 624
- Parabens, 435
- antibacterial activity, 293–296
 - antifungal activity, 296–297
 - application methods, 298–299
 - applications, 298–299
 - assays, 300–301
 - chemistry, 291–293
 - mechanism of action, 297–298, 639
 - metabolites, 300
 - regulation, 298
 - resistance development, 298
 - toxicology, 299–300
- Parasites, chlorine and, 515
- Parma ham, 209
- Parsley, 96
- Pasteurization methods
- high temperature/short time (HTST), 475
 - nonthermal, 248
- Pasteurized milk ordinance, 610
- Pasteur, Louis, 397
- Patulin, 155, 277
- Peaches, 624
- Pectin
- digestion, 343, 346
 - lysozyme inhibition, 367
- Pectinatus frisingensis*, 256
- Pediocins, 391, 392, 399, 402–406
- electroporation and, 623–624
 - and lactates, 119
 - production of, 398–399
 - resistance development, 399
- Pediococcus*, 149, 390
- bacteriocins, 402–406
 - diacetyl production, 592
 - sorbates and, 55
- Pediococcus acidilactici*, 208, 398
- bacteriocins, 403–406
 - diacetyl and, 592
- Pediococcus cerevisiae*, 149
- bacteriocins, 403
 - chlorine and, 516
 - nitrite and, 191
- Pediococcus dammosus*, 548
- Pediococcus paroulus*, 150
- Pediococcus pentosaceus*, 149, 398
- bacteriocins, 402–403
 - nitrite and, 204, 208
- Penicillic acid, 277
- Penicillin, 465
- discovery of, 361, 662
 - residue screening, 606
- Penicillin G, and monolaurin, 348
- Penicillium*
- benzoic acid and, 17
 - in cheese, 62
 - fruit spoilage, 154
 - monolaurin and, 343
 - mycotoxin production, parabens and, 296–297
 - sorbates and, 54, 57
- Penicillium chrysogenum*, 16, 126
- Penicillium cintrinum*, 281
- Penicillium corylophilum*, 122
- Penicillium cyclopium*, 109, 277
- Penicillium digitatum*, 515
- Penicillium discolor*, 280
- Penicillium expansum*
- citric acid and, 109
 - sodium diacetate and, 103
 - sulfur dioxide and, 154
- Penicillium fragi*, 115
- Penicillium glabrum*, 60
- Penicillium glaucum*
- acetic acid and, 99
 - dimethyl/diethyl dicarbonate and, 310
- Penicillium patulum*, 277
- Penicillium puberulum*, 577
- Penicillium roqueforti*, 63
- Penicillium thomii*, 310, 312
- Penicillium verrucosum* var. *cyclopium*, 63
- 1, 3-pentadiene, 5, 57
- Pep5, 394
- Peppermint, 438
- Pepsin, lactoperoxidase and, 458
- Peptides, antimicrobial, 377
- Peptidoglycan, cell wall, 362, 364, 636
- Peperularia*, 54
- Peracetic acid, 644
- Perborates, 537
- Percarbonates, 537
- Perigo factor
- in cured meats, 179, 192, 182–185
 - in media, 193
 - nitrite/nitrate and, 198–199
- Perillaldehyde, -lysozyme, 376
- Periodontitis, 461
- Peroxides, 537–551
- inorganic, 537
 - organic, 537
 - tolerance to, 650
- Peroxyacetic acid (PAA), 99, 537
- antimicrobial activity, 543–545
 - concentration effects, 545
 - inhalation studies, 551
 - mechanism of action, 543

- organic matter and, 549–550
- pH and, 547
- stability of, 542
- temperature and, 547–548
- toxicity, 550–551
- water hardness and, 549
- Peroxyacids
 - fresh produce sanitization, 561
 - poultry sanitization, 558
 - red meat sanitization, 555
- Peroxyesters, 537
- Peroxyoctanoic acid, 537
- Persimmons, 154
- Persulfates, 537
- Pestalotiopsis*, 54
- Pesticides
 - residues, 599
 - resistance development and, 616
- Pet foods
 - semi-moist, 591
 - sorbates and, 67
- pH
 - acid-anionic surfactants and, 534
 - and antimicrobial activity, 6
 - bacteria and, 92
 - chlorine/chlorine compounds and, 516–519
 - EDTA and, 636
 - and heat, 623
 - homeostasis, 124
 - hydrogen peroxide and, 540
 - iodophors and, 524–527
 - and lactoperoxidase, 457–458
 - microbial growth, 92
 - molds and, 92
 - and nitrite-sorbic acid mixtures, 196
 - organic acids and, 21, 640
 - peroxyacetic acid and, 547
 - phenolics and, 441–442
 - QACs and, 531–532, 635
 - and sorbic acid/sorbate, 58
 - yeasts and, 92
- Pharmaceuticals, sorbates and, 67
- Phenol, as a sanitizer, 435
- Phenolic coefficient technique, 435
- Phenolics, 291
 - applications, 442–444
 - classification, 426
 - mechanism of action, 436, 639
 - mycobacteria and, 644
 - pH and, 441–442
 - phenolic coefficient, 435
 - plant sources, 440–441
 - and proteins, 443
 - safety, 444–445
 - spores and, 643
- Phenoxyethanol, 640
- Phenylethanol, 639
- Phenylethylamine, 151
- Phenylformic acid, 12
- Phoma*, 54
- Phoma glomerata*, 282
- Phosphates
 - nitrite and, 197
 - and sorbates, 60
- Phosphatidylglycerol, 244
- Phosphofructokinase, 16
- Phospholipids, 244
- Phosphoroclastic oxidoreductase, 201
- Phytoalexins, 439–440
- Phytophenolic compounds, 291
- Pichia*, 54
 - salt tolerance, 577
 - sulfur dioxide and, 151
- Pichia membranaefaciens*, 23
- Pickles, 94, 98
 - softening of, 577
- Pigments
 - green, cured meat, 216
 - red, in ham, 209
- Pimaracin, 275
- Pimiento, 438
- Pineapple juice, 283
- Pirlimycin, 612
- Piscicolins, 399, 411
- Pizza, 257–258
- pKa, organic acids, 93
- Plantaricins, 391, 392, 409
- Plant oil extracts, parabens and, 295
- Plants, as antimicrobials sources, 429–446
- Plasma cells, 472
- Plasma membrane pumps, 70–71
- Plasmids
 - bacteriocins and, 392, 411
 - resistance and, 646–647
- Plasmolysis, cellular, 577
- Plasticizers, in aquatic systems, 615
- Pneumococcus*, 328, 483
- Poliovirus, 468, 515
- Poly(ADP) ribose polymerase, 17
- Polycations, 636
- Polyethylene films, parabens in, 299
- Polyethylene glycol, and lysozyme, 374
- Polyethyleneimines, 636
- Polyglycerol esters, 336
- Polymerase chain reaction (PCR), bacteriocins assay, 398
- Polymorphonucleocytes, lactotransferrin and, 461, 466
- Polyol, 522
- Polyphosphates, 581
 - antibotulinal effects, 197
 - antimicrobial activity, 580
 - applications, 581–582
 - assays, 583
 - chemistry, 578–579
 - combination treatment, 583
 - functions, 578
 - and lysozyme, 583
 - mechanism of action, 580–581
 - and nisin, 583
 - and pyrophosphates, 583
 - regulation, 583
- PolyP kinase, 581
- Polysaccharide, -lysozyme, 376
- Polyvinyl acetate coating, 280
- Polyvinylpyrrolidone (PVP), 51, 522
- Population homeostasis, 624
- Pore formation
 - bacteriocins, 395
 - nisin, 244–245
- Porins, 464, 481
- Pork
 - botulinal challenge tests, 196–197
 - canned, spoilage, 187
 - curing, 174–177
 - lactic acid and, 114–115
 - and lactic acid salts, 117–118
 - organic acid treatment, 96–97
 - polyphosphates and, 582
 - sorbates and, 66
 - sulfites and, 150
- Potassium, leakage of, 637
- Potassium benzoate, 12, 29
- Potassium bicarbonate, 589
- Potassium bisulfite, 157
- Potassium dichromate, 367
- Potassium metabisulfite, 157
- Potassium nitrate, 172, 218–221
- Potassium sorbate, 20, 50–54; *see also* Sorbic acid/sorbates
 - and clove, 442
 - EDTA and, 584
 - lysozyme and, 367
 - and methyl paraben, 294–295
 - and natamycin, 281–282, 283
 - and quinic acid, 22
 - regulation, 74–75
 - and sausages, 65
 - and sodium acetate, 101–102
 - and vanillin, 442
- Potatoes, 108
 - cooked, 257
 - sorbates and, 676–8
 - sulfites in, 157
- Poultry
 - FSIS residue survey, 603–609
 - lactic acid and, 115, 119
 - polyphosphates washes, 582
 - processing, 98
 - sanitizers, 556–559
 - sodium diacetate powder, 104
 - sodium nitrite and, 189
 - sorbates and, 66
 - succinic acid and, 123
- Powdery mildew, 154
- Prawns, 30, 615
- Prelantibiotics, 393–394

- Preservation
and antimicrobials, 6–7
microbial stress and, 624–625, 628
natural, 623
traditional methods, 622
- Preservatives
chemical, 2
definition of, 3
function of, 3–4
resistance. *See* Resistance development
role of, 648
- Preserves
sorbates and, 64
sulfur dioxide and, 152
- Principe V, 390
- Proanthocyanins, 436
- Produce, fresh
acid rinses, 96
sanitizers, 559–562
- Profile1, 668
- Proflavine, 641
- Proline, 577
- 1, 2-propanediol, 591
- Propionate, and sorbate, 60
- Propionibacterium freudenreichii* ssp. *shermanii*, 413–414
- Propionibacterium jensenii*, 414
- Propionibacterium thoenii*, 413–414
- Propionic acid
and acetic acid, 121
antimicrobial activity, 120–122
applications, 123
interactions, 122
regulation, 123
salts of, 121
- Propionicin, 414
- Propylene glycol, 591
- Propylene oxide, 641
- Propyl gallate, 59–60, 298, 436
- Propyl paraben, 291–301, 349
- Proteins
acid amines and, 534
benzoic acid binding, 28
cytoplasmic, 641–642
dicarbonate and, 313
fatty acid activity, 483
in foods, 674
heat-shock, 56, 649
lysozyme interactions, 367
peroxide-inducible, 649
and phenols, 443
sorbates and, 72
stress and, 126–127
- Protein synthesis, chlorine dioxide and, 512
- Proteus*, 55, 472, 645
- Proteus vulgaris*, 121
- Protocatechuate pathway, 27
- Proton-motive force
dissipation of, 639–640
sorbates and, 69
- Protoplasts, 636, 639
- Protoporphyrin, 204
- Protozoa
lactotransferrin and, 467, 468
QACs and, 529
- Providencia stuartii*, 645
- Provolute, 375
- Pseudomonas*
benzoate metabolism, 27
diacetyl and, 592
polyphosphates and, 580
propionic acid and, 122
salt tolerance, 575
sodium lactate and, 116
sorbates and, 55, 66
sulfur dioxide and, 150
- Pseudomonas aeruginosa*
acetic acid and, 99
acid tolerance, 121
benzoate and, 16
citric acid and, 110
EDTA and, 584, 636
IgY and, 478–479
lactolipids and, 483
monolaurin and, 338
nitrite and, 192
parabens and, 294, 298
QACs and, 529, 531
resistance development, 646
- Pseudomonas agglomerans*, 298
- Pseudomonas cepacia*, 33
- Pseudomonas fluorescens*
benzoic acid and, 12
EDTA and, 584, 586
iodophors and, 523
monolaurin and, 338
soaps and, 329
sodium diacetate and, 103
- Pseudomonas fragi*, 103
EDTA and, 586
monolaurin and, 338
parabens and, 293
- Pseudomonas phaseolicola*, 346
- Pseudomonas putida*, 26–27, 294–295
- Pseudomonas putrefaciens*, 586
- Pseudomonas solanacearum*, 27
- Pullularia*, 54
- Purine, chlorinated, 556
- Putrescine, 151
- Pyrimidines, chlorinated, 556
- Pyrophosphates, 245, 578, 579
and polyphosphates, 583
- Pyruvate, 147
- Pyruvate oxidoreductase, 202
- Pyuria, 37
- Q**
- Quaternary ammonium compounds (QACs)
antimicrobial activity, 529–531
chemistry, 528–529
gram-negative bacteria and, 529, 635, 645
mechanism of action, 529, 637–638, 639
mycobacteria and, 644–645
organic matter and, 532
pH and, 531–532, 635
resistance development, 531–532, 648
spores and, 643
temperature and, 532
toxicity, 532–533
water hardness and, 532
- Queso fresco, 63, 251
- Quinolones, 646
- R**
- RABIT, 670
- Raffinose, 146
- Rambutans, 154
- Raw materials, decontamination, 625
- Reactive oxygen species, nitric oxide and, 220
- Reduced-calorie mayonnaise (RCM), 95
- Refermentation
champagnes, 152
yeasts, 64–65
- Relishes, sorbates and, 64
- Residues. *See* Antibiotic/Antimicrobial residues
- Resistance development
acquired, 26, 646–647
antibiotic/antimicrobial residues and, 615–616
antimicrobials, 9, 642–647
bacteriocins, 399–400
biocides, 642–647
chlorine/chlorine compounds, 512–516
fatty acids, 346
gram-negative bacteria, 645
and hurdle technology, 628
intrinsic, 26
lactotransferrin, 465
lysozyme, 368–369
molds, 645
mutational, 646
mycobacteria, 644–645
nisin, 245–246
organic acids, 22–26, 126–127
parabens, 298
plasmid-mediated, 646–647

- QACs, 531–532, 648
 sessile bacteria, 647
 yeasts, 645
- Respiratory syncytial virus, 468, 483
- Response regulator, 395
- Reuterins, 407
- Reutericyclin, 407
- Reuterin, 407
- Reversed-phase high-performance liquid chromatography (RP-HPLC)
 fatty acids analysis, 482
 lysozyme, 364
 sodium benzoate, 37
 sorbic acid, 74
- Rhinoviruses
 fatty acids and, 336
 nitric oxide and, 220–221
- Rhizoctonia*, 54
- Rhizopus*, 54
 benzoate metabolism, 27
 fruit spoilage, 154
- Rhizopus nigrificans*, 99
- Rhizopus stolonifer*, 154
- Rhodia, Inc., 415
- Rhodococcus*, 27
- Rhodotorula*, 54, 151
 essential oils and, 438
 salt tolerance, 577
- Rhodotorula rubra*, 468
- Rice, fermented, 210
- Richardson, B.W., 538
- Ricinelaideate, 329
- Ricinoleate, 328, 329, 331
- Ricinstearolate, 329
- Ricotta, 251
- Rimodicin, 276
- Ringworm, 331
- Rinses
 acid, 96, 97
 lactic acid, 112, 115
 preevisceration, 97
- Ripper method, 159
- RNA, leakage of, 637
- Rock salt, 574
- Rope bacteria, 120, 149
- Roquefort cheese, 62
- Rosellinia*, 54
- Rosemary, 438
- Rotavirus, 468, 479
- Rowanberries, 49
- S**
- Sabinene, 444
- Saccharomyces*
 organic acids resistance, 24
 sorbates and, 54, 56
 sulfur dioxide and, 151–152
- Saccharomyces bayanus*, 12, 20
- Saccharomyces bisprus*, 110
- Saccharomyces cerevisiae*
 acetic acid and, 94
 benzoic acid/benzoate, 15, 23
 biocide susceptibility, 645
 chlorine and, 516
 DHA and, 104
 dimethyl/diethyl dicarbonate and, 312
 ethanol and, 590
 growth modeling, 627
 lactolipids and, 484
 lysozyme and, 371, 378
 natamycin and, 283
 organic acids testing, 36
 peroxyacetic acid and, 548
 sodium bicarbonate and, 588–589
 sorbate resistance, 56
 sulfur dioxide and, 148, 151–152
 thermotolerance, 15, 59
- Saccharomyces ellipsoideus*, 99, 121
- Saccharomyces ludwigii*, 151–152
- Saccharomyces rouxii*
 acetic acid and, 95
 citric acid and, 110
 sorbates and, 58
- Safety, antimicrobials, 7–8
- Sage, 438
- St. John's Wort, 617
- Sakacins, 391, 392, 398–399, 410
- Sake, 317, 366, 375
- Salad dressings, 67, 257
- Salad greens, 560
- Salami, 283, 375
- Salicylate, 27
- Salicyldehyde, 438
- Saliva, 220, 362
- Salmon
 cold smoked, 211–212, 255
- Salmonella*
 acetic acid and, 95, 96, 97, 98
 ethanol and, 590
 IgY and, 478–479
 lactic acid and, 114–115
 lactotransferrin and, 470
 nitrite and, 212, 213, 214–215
 organic acids and, 31–32
 polyphosphates and, 582
 salt tolerance, 576
 sodium lactate and, 116
 sorbates and, 55, 63, 66
 SOS response, 650
 sulfites and, 150
- Salmonella Aertrycke*, 94
- Salmonella Anatum*, 109, 120, 212
- Salmonella Blockley*, 94
- Salmonella Enteritidis*
 acetic acid and, 96–97
 citric acid and, 108–109
 lactolipids and, 483
 lysozyme and, 370
 salt concentration and, 213
- sodium diacetate and, 103, 104
- Salmonella* Montevideo, 55, 59, 560
- Salmonella* Oranienburg, 109
- Salmonella* Senftenberg
 acid tolerance, 120–121
 lysozyme and, 370
 nitrite and, 214
- Salmonella* Tennessee, 121
- Salmonella* Typhi, 121
- Salmonella* Typhimurium
 acetic acid and, 96, 97, 98
 acid-anionic surfactants and, 534
 adaptive response, 126–127
 cetylpyridinium chloride and, 559
 chlorine and, 512
 citric acid and, 110
 diacetyl and, 592
 DT104, 59
 iodine and, 527
 lactic acid and, 112, 113–114, 115, 117–119
 in luncheon meat, 212–213
 lysozyme and, 368, 370
 lysozyme-EDTA and, 373
 monolaurin and, 341, 342
 nisin and, 253
 nitrite and, 179, 213–216
 organic acids and, 557
 succinic acid and, 123
 trisodium phosphate and, 559
- Salmonellosis, cured meats, 215
- Salsa, 318
- Salt, 574–578
 curing. *See* Curing salts
 and essential oils, 442
 lysozyme and, 367
- Salting, 622
- Salt tolerance, bacteria, 575–577
- San Daniele ham, 209
- Sanitation, 7
- Sanitizers
 acid-anionic surfactants, 533–536
 amphoteric surfactants, 536–537
 fatty acid, 533–536
 food tissue surfaces, 552–562
 halogens, 509–522
 iodophors, 522–528
 no-rinse, 529
 peroxides, 537–551
 peroxyacetic acid, 542–551
 quaternary ammonium compounds, 528–533
 regulation, 551
- Saponin, 330
- Sarcina*, 368
- Sarcina lutea*, 121
- Sardines, 30
- Sauces, meat, 255
- Sauerkraut, 94
- Sausage
 blood fractions, 206

- curing brine, 174
dry
 sorbates and, 65
 starter cultures, 208
fatty acid preservatives, 340
fermented, 191–192, 213
 botulinal challenge tests, 196
and lactic acid salts, 117–118
monoacylglycerols in, 484
natamycin and, 283
nisin and, 254
nitrite and, 204, 216
pediocins and, 404
polyphosphates and, 582
sorbates and, 54
 Vienna, 183
Savoie ham, 209
Savory, 437
Scald water
 acetic acid in, 98
 lactic acid in, 115
 nisin in, 253
Sceptor, 668
Schizosaccharomyces japonicus, 152
Seafood
 lactotransferrin and, 468
 lysozyme and, 375
 monolaurin and, 343
 nisin and, 255
 nitrite/nitrate and, 210–212
 residues in, 614–615
Seminal fluid, lactotransferrin in, 460, 461
Semliki forest virus, 483
Sensititre, 668
Septicemia, lactotransferrin levels, 461
Serine, parabens and, 297
Serratia, 55, 214
Serratia liquefaciens, 66
 and lactic acid salts, 118
 polyphosphates and, 582
 soaps and, 329
Serratia marcescens, 150
 parabens and, 297
 resistance development, 646
Sewage, and drug residues, 615
Sheep, FSIS residue survey, 603–609
Shelf life, long-term, 2
Shellfish, nisin and, 255
Shewanella putrefaciens, 103
Shigella, 106
Shigella flexneri
 nitrite and, 215
 salt tolerance, 576
Shogaol, 444
Shrimp, 67, 102
Shugar unit, 363
Siderophores, 464
Sigma factor M, 650
Silver, 640, 647
Silver ion chromatography, fatty acids
 analysis, 482
Sinapic acid, 436
Skin
 hydrogen peroxide applications, 542
 peroxyacetic acid and, 551
 sorbic acid and, 72
Slaughter class, FSIS residue survey, 601
Smoking, 430, 622
Soaps, *see also* Fatty acids
 antibacterial, 328–330
 history, 327–328
 saturated, 329
 unsaturated, 329
Sodium 1-octane sulfonate, 533
Sodium abetate, 331
Sodium acetate, 101–103
Sodium acid pyrophosphate, 578
Sodium alginate, 119
Sodium ascorbate, 190
Sodium azide, 347
Sodium benzoate, 11, 12; *see also*
 Benzoic acid/benzoate
Sodium bicarbonate, 588–589
Sodium bisulfite, 157, 640
Sodium chloride
 antimicrobial activity, 575–577
 applications, 577–578
 assays, 578
 chemistry, 574
 mechanism of action, 577
 production, 575
 safety, 578
Sodium citrate, 109
Sodium dehydroacetate, 104–105
Sodium diacetate, 103–104
Sodium dichloroisocyanurate, 515, 521
Sodium dioctylsulfosuccinate, 533
Sodium dodecylbenzene sulfonate, 533
Sodium dodecyl sulfate, 363
Sodium erythorbate, 120, 189
Sodium hexametaphosphate, 581–583, 636
Sodium hypochlorite, 515
Sodium isoascorbate, 186–187, 190
Sodium lactate, 103–104, 116–120
Sodium lauryl sulfate, 533
Sodium metabisulfite, 157, 246, 640
Sodium nitrite, 20, 218, 367
Sodium nitroprusside, 202
Sodium peroxide, 537
Sodium propionate, 121
Sodium sorbate, 50–54; *see also*
 Sorbic acid/sorbates
Sodium sulfite, 157, 640
Sodium tripolyphosphate (STPP), 580–583
Sodium undecylenate, 329
Sodium xylenesulfonate, 533–534
Soft drinks
 benzene levels, 28
 dimethyl/diethyl dicarbonate and, 318
 sorbates and, 64
 sulfites in, 149
Soil Association, 614
Solvents, polar, 435
Sorbamide, 51
Sorbates. *See* Sorbic acid/sorbates
Sorbic acid/sorbate, 5
 allergic-type response, 72
 antimicrobial activity, 54–61
 application methods, 61, 63
 applications, 61–68
 assays, 73–74
 bakery products and, 65
 and benzoate, 14–15
 and bisulfite, 147
 chemistry, 50–54
 and cinnamon, 442
 combinations, 59–60
 commercial applications, 53
 dairy products and, 61–63
 degradation, 51, 57
 effect of solutes, 58
 and fruit products, 64–65
 heat and, 58–59
 inhibitory action, 54–55
 interactions, 57–61
 manufacture of, 53–54
 meat products and, 65–67
 mechanism of action, 68–71
 MIC, 57
 and monolaurin, 340–342
 mutagenicity, 72–73
 -nitrite reactions, 72
 pH and, 58
 regulation, 74–75
 selectivity, 55–56
 sodium benzoate and, 33
 solubility, 50–53
 sources, 49–50
 storage temperature and, 59
 toxicology, 71–73
 uses, 50
 and vanillin, 60
 and vegetable products, 63–64
Sorbic anhydride, 51
Sorbhydroxamic acid, 51
Sorboyl CoA, 74
Sorboyl palmitate, 51, 65
Sorbyl peroxides, 69
SOS response, 650
Soups, chilled, 256
Soy products, nisin and, 257
Soy proteins, 188
Soy sauce, 340, 484
Spectinomycin, 612

- Spectrometry
 benzoate, 37
 natamycin, 286–287
- Spectrophotometry
 dimethyl/diethyl dicarbonate, 320
 natamycin, 287
 parabens, 300
 sorbic acid, 73
 turbidity changes, 671–672
- Spheroplasts, 636, 639
- Spices, as antimicrobials sources,
 429–446
- Spiral gradient endpoint (SGE) test,
 667
- Spiral plating, 667–668
- Spleen focus forming virus, 468
- Spore-formers, sorbates and, 56
- Spores
 biocides and, 635
 chlorine and, 512–516
 coats, 635, 643
 germination, 68
 heat-resistant, 241–244
 hydrogen peroxide and, 539
 iodophors and, 523
 lysozyme and, 368, 369–370
 nisin and, 241–244, 246
 peroxyacetic acid and, 544, 547
 phenolics and, 438
 resistance development, 643
 sorbate and, 68
- Sporicids, 635, 643
- Sporistats, 643
 chlorine, 512–516
 phenolics, 438
- Sporobolomyces*, 54
- Sporolactobacillus*, 56, 202
- Sporotrichum*, 54
- Sporulation, bacterial, 650
- Spot-on-lawn method, 664
- Sprays
 acetic acid, 553
 acidified sodium chlorite, 56–557
 antimicrobial, 552
 cetylpyridinium chloride, 559
 citric acid, 553
 lactic acid, 112–114, 119, 553
 organic acid, red meat sanitization,
 553
 peroxyacids, 558
 sodium bicarbonate, 588
 succinic acid, 123
- Spreads, microstructure, 627
- Staphylococcus*, 55
 nitrate reduction, 208
 salt tolerance, 575–576
 soaps and, 328
- Staphylococcus aureus*
 acid tolerance, 115
 in bacon, 189
 benzoate and, 20
 biocide susceptibility, 645
 chlorine and, 512–514
 citric acid and, 109–110
 EDTA and, 585, 636
 enterotoxin production
 polyphosphates and, 580–581,
 582
 salt concentration and, 576
 ethanol and, 590
 IgY and, 478–479
 lactolipids and, 483
 lactotransferrin and, 461
 lysozyme and, 368, 370
 malic acid and, 120
 methicillin-resistant, 648
 nitrite/nitrate and, 175, 191–192,
 200
 organic acids and, 32, 94, 95
 parabens and, 293–294, 297–298
 resistance encoding plasmids, 646
 soaps and, 329
 sodium lactate and, 116, 118
 sodium propionate and, 121
 sorbic acid/sorbates and, 55, 60,
 66, 69
- Staphylococcus carnosus*, 209
- Staphylococcus caseolyticus*, 209
- Staphylococcus epidermidis*
 epidermin, 391
 gallidermin, 391
 lactolipids and, 483
 propylene glycol and, 591
- Staphylococcus lentus*, 209
- Staphylococcus warneri*, 209
- Starch, 330, 335
- Starter cultures
 lactoperoxidase in, 459–460
 lysozyme and, 371
 mixed-strain, 408
- Starvation, bacterial, 649, 650
- Steam vacuuming, 625
- Stearate, 327, 329, 331
- Stemphyllum*, 154
- Sterols, polyene macrolides and, 280
- Strawberries, 64, 105, 318
- Streptacidin, 479
- Streptavidin, 480
- Streptococcus*, 390
 diacetyl production, 592
 lactolipids and, 483
 lactostreptins, 400
 soaps and, 328
- Streptococcus agalactiae*, 109, 343
- Streptococcus diacetylactis*, 411–412
- Streptococcus durans*, 179
- Streptococcus faecalis*
 in liver sausage, 204
 nitrite and, 179, 192, 202
 organic acids sensitivity, 95
- Streptococcus faecium*, 179
- Streptococcus iniae*, 343
- Streptococcus lactis*, 192, 516
- Streptococcus mutans*, 391, 478–479
- Streptococcus salivarius* var.
 thermophilus, 63
- Streptomyces*, bacteriocins, 391
- Streptomyces chattanoogensis*, 275
- Streptomyces cinnamoneus*, 391
- Streptomyces natalensis*, 275, 277
- Streptomyces rutgerensis*, 375
- Streptomycin, 606, 612
- Streptovercillum*, 391
- Stress response, 126–127, 649–651
 and food preservation, 624–625,
 628
 and resistance development, 9
- Subtilin, 392
- Subtilopeptidase, 247
- Succinate, 27
- Succinate dehydrogenase, 16
- Succinic acid, 123
- Succinic dehydrogenase, 69
- Sucrose, 146, 636
- Sucrose esters, antimicrobial activity,
 336
- Sugar-based products, sorbates and,
 67
- Sugars, bisulfite reactions, 146
- Sulfadiazine, 606
- Sulfadoxime, 606
- Sulfamerazine, 606
- Sulfamethazine, 606
- Sulfamethiazole, 606
- Sulfamethoxazole, 606
- Sulfanilamides, 328
- Sulfa-on-Site (SOS), 602, 603
- Sulfapyridine, 606
- Sulfaquinolaxaline, 606
- Sulfate permease, 152
- Sulfates, 143
- Sulfite oxidase, 157
- Sulfites. *See* Sulfur dioxide/sulfites
- Sulfite test strips, 159
- Sulfonamides
 in aquatic systems, 615
 residues, 602, 603, 606–609
 residues in eggs, 614
 residues in milk, 609–612
- Sulfonates, antimicrobial activity, 148
- Sulfur, 143
- Sulfur dioxide/sulfites, 143, 144–148,
 157, 640–641
 adverse reactions, 156
 antagonistic effects, 21
 antimicrobial activity, 148–155
 assays, 158–159
 browning prevention, 155
 and dimethyl dicarbonate, 310
 forms of, 144–145
 and molds, 154–155
 molecular, 151
 reactivity, 145–148

- regulations, 157–158
 - solubility, 144
 - and sorbates, 64
 - sources, 143–144
 - toxicology, 155–157
 - uses, 143
 - Sulfurous acid
 - antimicrobial activity, 148
 - measuring, 158–159
 - Sulfur oxide, 143
 - Sunset yellow, 147
 - Superoxide, 220, 538–539
 - Surfactants, 528–536
 - acid-anionic, 533–536
 - amphoteric, 536–537
 - anionic, 637–638
 - lysozyme and, 366
 - nisin and, 248
 - nonionic, benzoic acid and, 21
 - quaternary ammonium
 - compounds, 528–533
 - Swab Test on Premises (STOP), 602, 603
 - Sweet marjoram, 438
 - Swine, FSIS residue survey, 603–609
 - Swiss cheese, 123, 281, 538
 - Synergism
 - antimicrobial combinations, 9, 673–674
 - organic acids, 20–22
 - Syrups, 64
- T**
- Talaromyces flavus*, 23, 111
 - Taleggio, 370
 - Tall oil fatty acid, 534
 - Tannins, 436
 - Target sites
 - antimicrobials, 634–635
 - mutations of, 646
 - Tartaric acid, 124
 - Tea, 436
 - Tears
 - lactotransferrin in, 460, 461
 - lysozyme in, 362
 - Teat dip, 338
 - Teewurst, 213
 - Tego, 536–537
 - Temperature
 - and acetic acid, 95
 - chlorine/chlorine compounds and, 519
 - hydrogen peroxide and, 540–541
 - and immunoglobulin stability, 475
 - iodophors and, 527–528
 - and lactoperoxidase, 457–458
 - nisin and, 247
 - peroxyacetic acid and, 547–548
 - QACs and, 532
 - Tenecetin, 275
 - Teratogenicity, sodium benzoate, 37
 - Tert-butyl hydroxyanisole (BHA),
 - fatty acids and, 349
 - Tertiary butyl hydroquinone (TBHQ),
 - 59–60, 298, 436
 - Tetrachlorosalicylanilide, 347, 348
 - Tetracycline, 606
 - in aquatic systems, 615
 - residues in eggs, 614
 - residues in milk, 610, 612
 - resistance development to, 646
 - Tetragenococcus*, 390
 - Tetrahymena gelei*, 329
 - Tetrin A, 276
 - Tetrin B, 276
 - Thaumatins, 440
 - Thenard, Louis-Jacques, 538
 - Thermal processing
 - and acidification of foods, 107–108
 - and lysozyme stability, 366–367
 - Thermolysin, lactoperoxidase and, 458
 - Thermonuclease, 55
 - Thermophiles
 - acid blanching and, 108
 - nisin and, 623
 - Thermotolerance
 - ascospores, 109
 - organic acids and, 15
 - and sorbic acid/sorbate, 58–59
 - Thiamine, 147
 - Thiamine pyrophosphate (TPP), 148, 201
 - Thin-layer chromatography, parabens, 300
 - Thiocyanate, lactoperoxidase and, 456–460
 - Thiol groups, interactions, 640
 - Thionins, 440
 - Thiosulfonates, 158
 - Thyme, 435, 436, 437
 - Thyme oil, 439
 - Thymol, 9, 436, 437–438
 - antimicrobial activity of, 432
 - heat and, 623
 - nisin and, 248
 - pH and, 441–442
 - Thyroperoxidase, 457
 - Time-kill curves, 670–671
 - combined antimicrobial testing, 673
 - interpretation, 677
 - Titanium dioxide, nisin and, 246
 - Titration, EDTA, 587
 - Tobacco smoke, NDMA in, 219
 - Tofu, 257
 - Tomatoes
 - acidification of, 107
 - benzoic acid in, 12
 - sorbates and, 64
 - sulfur dioxide and, 154
 - Tomato juice
 - fumaric acid and, 111
 - organic acids and, 31
 - sorbates and, 64
 - Toothpaste, lactoperoxidase systems, 460
 - Tortillas, 284
 - Torula*, 121
 - Torulaspora*, 54
 - Torulaspora delbrueckii*, 297
 - Torulopsis*, 54, 56, 151
 - Torulopsis glabrata*, 438
 - Torulopsis versatilis*, 95
 - Touch-SCAN/AutoSCAN, 668
 - Toxic shock, 348, 466
 - Toxin neutralization, 473, 475
 - Toxoplasma gondii*, 467, 468
 - Transferrin, 198, 460–472
 - serum, 460
 - Transposons
 - bacteriocins, 392
 - nisin, 258
 - Triacylglycerols, 482
 - Trichloroisocyanuric acid, 515–516
 - Trichlorophenol, 348
 - Trichoderma*, 54
 - Trichoderma interdigitale*, 331
 - Trichoderma purpureum*, 331
 - Trichoderma viride*, 330, 484
 - Trichophyton*
 - benzoic acid and, 19
 - lactotransferrins and, 468
 - Trichophyton mentagrophytes*
 - fatty acids and, 330
 - propionic acid and, 122
 - QACs and, 531
 - Trichothecium roseum*, 154
 - Triclosan, 638, 648
 - Triganopsis*, 56
 - Triglycerides, milk, 483
 - Trihalomethane (THM), 521
 - Trihydroxystearate, 331
 - Trimethylamine-N-oxide reductase, 16
 - Trimethylamineoxide (TMAO), 210
 - Triphenylmethane dyes, 641
 - Tripolyphosphates, 579
 - Trisodium phosphate
 - and lysozyme, 374
 - poultry sanitization, 558–559
 - red meat sanitization, 56
 - Tritrichomonas foetus*, 467, 468
 - Tropical fruit, hurdle technology and, 624
 - Trout, 255
 - Truncatella*, 54
 - Trypsin
 - lactoperoxidase and, 458
 - lactotransferrin and, 469
 - Tryptamine, 151

- Tryptone, 199
 Tumor metastasis, lactotransferrins and, 468
 Tumor necrosis factor (TNF), lactotransferrin and, 466
 Turbidimetry, 363, 671–672
 Turkey
 ovoglobulins, 476
 sodium diacetate and, 104
 and sodium lactate, 119
 sodium propionate and, 121
 Turmeric, 438
 Tylosin, residues in milk, 612
 Tyramine, 151
- ## U
- Ulcers, acetic acid-associated, 99
Ulocladium, 54
 Ultra-filtration, lactotransferrin, 462
 Ultrahigh hydrostatic pressure (UHP), 248, 623–624
 Ultra-high temperature (UHT) processing, 241, 251
 Ultraviolet radiation, lysozyme inactivation, 367
Uncinula, 154
 Undecylenic acid, 331
 Underprocessing, 627–628
 Union of Concerned Scientists, 599
 Urea synthesis, congenital errors in, 36
 Urinary tract, 37
 U.S. Department of Agriculture (USDA)
 canned cured meat products, 175–176
 Expert Panel on Nitrites and Nitrosamines, 217
 Food Safety Inspection Service (FSIF), 97
 Pathogen Modeling Program, 206
 residue survey (FSIS), 600–609
 U.S. Food and Drug Administration (FDA). *See* Food and Drug Administration
 U.S. Geological Survey, 615
- ## V
- Vacuum packaging
 cured meats, 177
 franks, 188
 safety of, 179–180
 and *S. aureus*, 191
Vagococcus, 390
 Vancomycin, 465
 Vanillic acid, 436
 Vanillin, 436, 438
 antimicrobial activity of, 432
 fruit products, 443–444
 pH and, 442
 and potassium sorbate, 60, 442
 Variable region, immunoglobulins, 474
 Veal, lactic acid sprays, 112
 Vegetables
 benzoic acid and, 32–33
 canned, nisin and, 252–253
 fermented
 off-odors in, 57
 salt and, 577
 sorbates and, 56, 64
 lactic acid rinses, 115
 lysozyme and, 370, 375
 pickled, 64
 raw, 559–562
 sorbates and, 54, 56, 63–64
 Vegetarian products, nisin and, 257
 Vegetative cells, nisin and, 244–245
 Versene, 371, 583; *see also* under EDTA
 Vesicular stomatitis virus, 483
 Veterinary Medicine Directorate, 614
Vibrio, 55
 benzoate metabolism, 27
 lactotransferrin and, 464
 salt tolerance, 575
Vibrio parahaemolyticus
 benzoate and, 19
 caprylic acid and, 106
 iodophors and, 523
 monolaurin and, 335
 salt tolerance, 576
Vibrio vulnificus
 benzoate and, 19
 diacetyl and, 592
 sorbic acid and, 58
 Vienna sausages, 183, 375
 Vinegar, 94, 149
 Viruses
 chlorine and, 514–515
 defensins and, 483, 485
 fatty acids and, 483
 hydrogen peroxide and, 539
 IgY and, 478–479
 iodophors and, 523
 lactoperoxidase and, 459
 lactotransferrins and, 465, 466–467, 468
 neutralization, 473, 475
 peroxyacetic acid and, 544
 QACs and, 531
 Visna virus, 483
 von Hoffman, A.W., 49
 von Leeuwenhoek, Antonie, 397
- ## W
- Wash water
 curd, 282
 lactic acid in, 113
 Water
 chlorine in, 521
 drug residues in, 615
 nitrite in, 219
 oxygenated, 538
 ozonated, 556, 559
 solubility and antimicrobials, 5
 Water hardness
 acid-anionic surfacants and, 534
 chlorine/chlorine compounds and, 521
 iodophors and, 528
 peroxyacetic acid and, 549
 QACs and, 532
 Wedge plating, 666–667
Weissella, 390
 Wheat flour doughs, and sorbic acid, 53
 Whey, 456
 commercial, 476
 lactotransferrin in, 462
 Wine
 diacetyl and, 592
 dimethyl/diethyl dicarbonate and, 306, 309–310, 317, 321
 dry, 152
 ethanol fortification, 590
 lactic acid bacteria, 149
 lysozyme and, 375, 379
 malolactic fermentation in, 149–151
 natamycin and, 284
 nisin and, 256
 off-odors in, 57
 sorbates and, 54, 64–65
 sparkling, 305
 sulfite hypersensitivity, 156–157
 sulfites in, 149, 157, 158
 sulfur dioxide and, 146–148, 151–154
 Withdrawal times, antibiotics, 600
 Wrappers
 butter, 103
 cheese, 106
- ## Y
- Yarrowia lipolytica*, 14
 Yeasts, 472
 acid-anionic surfacants and, 534
 benzoic acid/benzoate and, 16, 17, 22–25
 brewing, 256
 chlorine and, 512–514
 dimethyl/diethyl dicarbonate and, 309–310
 essential oils and, 440
 fresh produce and, 559
 growth modeling, 627
 hydrogen peroxide and, 539

- iodophors and, 523
 - “killer,” 151
 - lactates and, 116–117
 - lactotransferrin and, 466
 - lysozyme and, 371
 - natamycin and, 277–279
 - nitrite and, 215–216
 - osmotolerant, 56
 - parabens and, 297
 - pH and, 92
 - QACs and, 529
 - quinic acid and, 22
 - refermentation of, 64–65
 - resistance development, 645
 - salt tolerance, 577
 - sorbates and, 54, 56
 - sulfur dioxide/sulfites and, 151–154
 - thermotolerance, 15
 - Yersinia*, 55
 - peroxyacetic acid and, 544
 - sodium lactate and, 116
 - Yersinia enterocolitica*, 103
 - acetic acid and, 95, 96
 - acid tolerance, 107
 - chlorine and, 512
 - citric acid and, 110
 - lactic acid and, 114–115, 118
 - lysozyme and, 368, 369
 - nitrite and, 215
 - parabens and, 294–295
 - sorbates and, 66
 - sulfites and, 150
 - Yogurt, 63
 - benzoic acid in, 12
 - lactoperoxidase and, 459–460
 - natamycin in, 284
 - Yolk, egg, IgY in, 477–479
- Z**
- Zeamatins, 440
 - Zinc, 617
 - EDTA chelation, 585
 - ovotransferrin, 472
 - Zingerone, 438, 444
 - Zygosaccharomyces*
 - organic acids resistance, 24
 - sorbates and, 54, 56
 - stress adaptations, 649–650
 - Zygosaccharomyces bailii*
 - benzoate and, 14–15
 - growth modeling, 627
 - molecular, 151–152
 - organic acids and, 23–24, 30
 - parabens and, 297
 - sorbic acid/sorbates and, 55, 56, 57, 60
 - Zygosaccharomyces bisporus*, 297
 - Zygosaccharomyces lentus*, 24
 - Zygosaccharomyces rouxii*
 - hurdle approach, 54
 - lysozyme and, 371
 - parabens and, 297
 - sorbates and, 59
 - Zylux, 668