

Naturally
Occurring
Antimicrobials
in Food



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Agricultural, and Environmental Issues

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Naturally Occurring Antimicrobials in Food

Council for Agricultural Science and Technology
Printed in the United States of America
Cover design by Lynn Ekblad, Different Angles, Ames, Iowa
ISBN 1-887383-12-3
ISSN 0194-4088
01 00 99 98 4 3 2 1

Library of Congress Cataloging in Publication Data

Naturally occurring antimicrobials in food.
p. cm. — (Task force report, ISSN 0194-4088 ; no. 132)
Includes bibliographical references and index.
1. Food additives. 2. Food preservatives. I. Council for
Agricultural Science and Technology. II. Series: Task force
report (Council for Agricultural Science and Technology) ; no.
132
TX553.A3N36 1998
664'.0287—dc21

97-33248
CIP

Task Force Report
No. 132 April 1998

Council for Agricultural Science and Technology

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Contents

Interpretive Summary	1
Food Preservation and Safety, 1	
Naturally Occurring Antimicrobials, 1	
Future of Natural Antimicrobials, 2	
Summary	3
1 Introduction,	9
2 Role and Need for Antimicrobials in Food,	12
3 Description of Antimicrobials,	15
Introduction, 15	
Traditional Antimicrobials, 17	
Organic Acid Antimicrobials, 18	
Introduction, 18	
Acetic Acid, 19	
Benzoic Acid, 19	
Lactic Acid, 20	
Propionic Acid, 21	
Sorbic Acid, 22	
Other Organic Acids, 23	
Lipid Antimicrobials, 25	
Introduction, 25	
Fatty Acids, 25	
Monoacylglycerols and Other Fatty Acid Esters, 26	
Lipopeptides, 28	
Antimicrobial Plant Substances, 28	
Introduction, 28	
Garlic and Onion, 32	
Phenolic Compounds in Spices and Herbs, 35	
Plant Pigments and Related Compounds, 37	
Other Plant Phenolic Compounds, 38	
Hops, 39	
Coffee, Tea, Kola, and Cocoa, 40	
Carbohydrates, 41	
Phytoalexins, 41	
Polypeptide Antimicrobials, 42	
Introduction, 42	
Lytic Enzymes, 44	
Peroxidases and Oxidases, 48	
Transferrins, 50	
Antimicrobial Peptides, 53	
Miscellaneous Polypeptides, 60	

Miscellaneous Microbial Metabolites with Antimicrobial Activity, 60	
Introduction, 60	
Ethanol, 61	
Natamycin, 62	
Nonprotein Inhibitors from Lactic Acid Bacteria, 63	
4 Interactions of Antimicrobial Hurdles	66
5 Activity and Uses in Foods	67
Introduction, 67	
Traditional Antimicrobials, 67	
Organic Acid Antimicrobials, 67	
Lipid Antimicrobials, 68	
Antimicrobial Plant Substances, 69	
Polypeptide Antimicrobials, 71	
Miscellaneous Microbial Metabolites with Antimicrobial Activity, 74	
6 Potential for Applications	77
7 Conclusions	80
Appendix A: Symbols, Acronyms, and Abbreviations	81
Appendix B: Glossary	82
Literature Cited	83
Index	99

Figures

- 2.1 *Trichoderma*, a fungal contaminant, on an orange, 12
- 2.2 A melon infected with several species of fungi, 13
- 3.1 Acids are important in the preservation of pickled, acidified, and fermented foods such as cucumber pickles, 16
- 3.2 Structures of the organic acids, acetic, propionic, lactic, and fumaric, produced by some microorganisms or naturally present in some foods, 18
- 3.3 Benzoic acid is an antimicrobial acid, which is a natural constituent of cranberries, prunes, green-gage plums, apples, strawberries, and ripe olives, 19
- 3.4 Strawberries are one of the fruits containing benzoic acid, 20
- 3.5 Propionates are added to breads where they are active inhibitors of molds and rope-forming bacteria, 21
- 3.6 Sorbic acid is an effective antimicrobial agent against a variety of yeasts, molds, and bacteria, 22
- 3.7 Citric and ascorbic acids are two of many naturally occurring acids in many fruits and vegetables that are antimicrobial, 23
- 3.8 Many fruits contain naturally occurring acids that have an antimicrobial effect, 23
- 3.9 Monolaurin, a fatty acid ester, that may provide an antimicrobial effect in foods, 27
- 3.10 Plant phenolic compounds that are associated with antimicrobial activity, 29
- 3.11 Isothiocyanates are stored in the cell vacuoles of plants in the family Cruciferae and have antimicrobial properties, 31
- 3.12 Cabbage is an example of a plant in the family Cruciferae that contains isothiocyanates, 31
- 3.13 Garlic, a plant in the *Allium* genus (*Allium sativum*), as well as onion and leek are probably the most widely consumed foods with substantial antimicrobial activity, 32
- 3.14 Garlic and other *Allium* species contain alliin, a precursor of allicin, which is released when bulb tissue is disrupted, 33
- 3.15 Cinnamon, which inhibits bacterial activity, mold growth, and mycotoxin production, is being stripped from the tree, 35
- 3.16 Rolls of cinnamon being dried, 35
- 3.17 Cinnamic aldehyde, thymol, and eugenol structures—antimicrobial compounds in spices, 35
- 3.18 The herb, sage, which contains the antimicrobial thymol, 36
- 3.19 The herb, mint, which contains the antimicrobial thymol, 37
- 3.20 Olives contain phenolic compounds that are antimicrobial, 38
- 3.21 Flowers of the hop vine, *Humulus lupulus*, are used to impart bitter flavors and antimicrobial properties to beer, 39

- 3.22 Caffeine, contained in coffee (above) and cocoa beans, is antimycotic as well as antibacterial. It also inhibits the production of mycotoxins, 40
- 3.23 Phytoalexins isolated from plants in the family Leguminosae, 41
- 3.24 Pisatin is an antimicrobial phytoalexin present in peas, 42
- 3.25 Structure of egg white lysozyme, an example of a lytic enzyme having antimicrobial properties, 44
- 3.26 Hens' eggs contain lysozyme, 45
- 3.27 Lactoferrin, a protein present in milk, exhibits antimicrobial activity, 51
- 3.28 Nisin, a bacteriocin, that acts against bacteria is approved for use in process cheese products, 54
- 3.29 Example of gas formation and late-blowing of cheese, 55
- 3.30 Meat and vegetable fermenting bacteria produce pediocin PA-1, which is effective against certain pathogenic bacteria, 57
- 3.31 Ethanol is an effective naturally occurring antimicrobial that has been recognized since the first alcoholic fermentations of fruits to produce wines, 61
- 3.32 Natamycin is active against nearly all molds and yeasts, 62
- 5.1 Benzoate may be used as a preservative in many foods including fruit juices, 68
- 5.2 Propionates and sorbates are used as preservatives in many foods such as bakery goods, 68

Tables

- 1.1 Classification of food preservation methods, 11
- 1.2 Common chemical antimicrobials used in foods in various countries, 11
- 2.1 Major natural antimicrobial systems and potential for synergy, 14
- 3.1 Antimicrobials found in food plants, 30
- 3.2 Isothiocyanate types, plant sources and their flavor/taste descriptors, 31
- 3.3 Plants used as spices and herbs that also contain compounds possessing antimicrobial activity, 37
- 3.4 Phenolic antimicrobial compounds found in some olive oils and associated materials, 39
- 3.5 Phytoalexin production by edible plants, 43
- 3.6 Biological properties of proteins of avian egg albumen, 45
- 3.7 Old and new names of lactic acid bacteria, 53
- 3.8 Antibacterial peptides found in insects, 59
- 5.1 Types of foods in which lysozyme has been used as a preservative, 71
- 5.2 Examples of microorganisms that have been used in preservation of foods or feeds around the world, 75

Foreword

Following a recommendation by the CAST National Concerns Committee, the CAST Board of Directors authorized preparation of a report on naturally occurring antimicrobials in food.

Dr. John N. Sofos, Department of Animal Sciences, Colorado State University, Fort Collins, served as chair for the report. A highly qualified group of scientists served as task force members and participated in the writing and review of the document. They include individuals with expertise in food science, food safety, and food toxicology.

The task force met and prepared an initial draft of the report. They revised all subsequent drafts of the report and reviewed the proofs. The CAST Executive and Editorial Review committees reviewed the final draft. The CAST staff provided editorial and structural suggestions and published the report. The authors are responsible for the report's scientific content.

On behalf of CAST, we thank the chair and authors who gave of their time and expertise to prepare this report as a contribution by the scientific community to public understanding of the issue. We also thank the employers of the scientists, who made the time of these individuals available at no cost to CAST. The members of CAST deserve special recognition because

the unrestricted contributions that they have made in support of CAST also have financed the preparation and publication of this report.

This report is being distributed to members of Congress, the White House, the U.S. Department of Agriculture, the Congressional Research Service, the Food and Drug Administration, the Environmental Protection Agency, the Agency for International Development, and the Office of Management and Budget, and to media personnel and institutional members of CAST. Individual members of CAST may receive a complimentary copy upon request for a \$3.00 shipping fee. The report may be reproduced in its entirety without permission. If copied in any manner, credit to the authors and to CAST would be appreciated.

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Interpretive Summary

Food Preservation and Safety

In recent years, advances in processing and distributing food have been associated with development and increased demand for minimally processed convenience products that are palatable, available throughout the year, and nutritious. At the same time, our society has experienced a centralization of food processing operations, decreased numbers of people involved in food production, increased urbanization, and increased world population. Furthermore, changes in lifestyles relative to food preparation and consumption have increased the potential for mishandling of foods during various stages of processing, storing, distributing, retailing, and preparing. Other modern developments have increased the life expectancy of humans, leading to an increasing proportion of immunocompromised individuals who are more susceptible to illness caused by microorganisms, some of which are resistant to adverse conditions such as refrigeration. For these reasons, food preservation to ensure food safety is becoming ever more critical to the survival and well-being of humans. The importance of food safety to our society is evidenced by major actions taken by federal, state, and local health and regulatory authorities in recent years. These include the new regulations for meat, poultry, and seafood inspection that are based on the principles of the hazard analysis critical control point (HACCP) system, as well as the President's National Food Safety Initiative, which was unveiled in 1997. These initiatives were undertaken in response to increased public concern about the safety of the United States food supply.

Naturally Occurring Antimicrobials

Among the approaches employed in achieving food preservation by inhibiting growth of undesirable microorganisms, is the use of chemical agents exhibiting antimicrobial activity. These chemicals may be either synthetic compounds intentionally added to foods or naturally occurring, biologically derived sub-

stances, i.e., *naturally occurring antimicrobials*. These substances may exhibit antimicrobial properties in the foods in which they normally are found or may be used commercially as additives to other foods requiring preservation.

Consumer perception that use of industrially synthesized food antimicrobials may be associated with potential toxicological problems has generated interest in the food industry for the use of naturally occurring compounds. It is important to note, however, that commonly used antimicrobials, such as organic acids that are routinely produced in large quantities through chemical synthesis, also are found naturally in many food products, and their toxicological safety as food additives is ensured by regulatory authorities. The extraction of these and other antimicrobials from natural sources, however, can be complex, inefficient, and expensive. Nevertheless, synthetic agents may be considered less desirable by a segment of the consuming public than are naturally occurring antimicrobials. Thus, interest in, and incentive for, development, and use of naturally occurring antimicrobials in foods have increased because of the growing interest in so-called *natural* foods. There also is potential for the use of antimicrobials to preserve foods destined for consumption, or produced, in developing countries, which have increasing needs for food but lack sufficient refrigeration and distribution systems.

Numerous naturally occurring antimicrobial agents are present in animal and plant tissues, where they probably evolved as part of their hosts' defense mechanisms against invasion by microorganisms. Natural antimicrobials can be derived from barks, stems, leaves, flowers and fruits of plants, various animal tissues, or from microorganisms. Noted sources of natural antimicrobials are herbs, spices, fruits, milk, eggs, and lactic acid bacteria used in food fermentation. However, selection, manufacture, and commercial application of a proper antimicrobial is challenging due to the complexity of food, the variety of factors influencing preservation, and the complex chemical and sensory properties of natural antimicrobials.

Future of Natural Antimicrobials

Naturally occurring antimicrobials could be useful as individual factors or hurdles in multifactor food preservation systems. This report discusses the chemistry, occurrence, activities, mechanisms of action, uses, application potential, and research and development needs of naturally occurring antimicrobials under the broad categories of lipids, plant substances, polypeptides, and microbial metabolites.

Of the many natural antimicrobials discussed in this report, only a few have been tested, or applied to foods. Examples of antimicrobials of natural origin that have been approved and have found certain uses include egg-white lysozyme, hydrogen peroxide, ethanol, the antibiotic natamycin, and the bacteriocin nisin. For more extensive use of natural antimicrobials, there is a need for research to examine their

- efficacy and functionality in models of food systems and foods,
- toxicology and safety in food formulations,
- interactions with food components and other preservative systems,
- mechanisms of action against microorganisms,
- influences on food quality (e.g., nutritional and sensory),
- methods for application in commercial formulations, and
- extraction, isolation, and economical production.

In summary, naturally occurring antimicrobials are abundant in the environment. The desire for expanded use is obvious, especially in light of consumer demands for minimally processed, safe foods of adequate shelf-life and convenience, and the global need for increasing the supply of food. For the use of naturally occurring antimicrobials to increase, however, research, commercial development, and economic production must occur on an adequate scale; sensory changes in foods must be avoided; food safety must be assured; multifactor preservative systems including natural antimicrobials must be developed; and technology transfer must occur. With availability of economical food preservation systems based on natural antimicrobials, the world will have an additional weapon in the struggle against hunger.

Summary

Growth of undesirable microorganisms in foods leads to products' becoming unfit for human consumption and contributes to worldwide food shortages, with all their morally and economically undesirable consequences. Prevention of losses of human life that result from food shortages, malnutrition, or microbial foodborne illness is a challenge demanding the utmost attention from the scientific community, public health agencies, and regulatory authorities. An important task of scientists involved in the study of food, as well as of the food industry and regulatory authorities, is to develop methods of preserving the food supply.

Food preservation can be achieved by (1) aseptic handling to prevent or minimize entry of microorganisms into food; (2) removing microorganisms through washing, centrifugation, or filtration; (3) destroying microorganisms with heat (i.e., pasteurization, canning) or irradiation; and (4) inhibiting growth of microorganisms through environmental control (e.g., refrigeration, freezing, drying, packaging), adding chemical antimicrobials, or by adding desirable microorganisms in fermented foods (e.g., cheeses, pickles) to compete with undesirable microorganisms.

Among the approaches to achieving food preservation is the use of chemical agents exhibiting antimicrobial activity. These chemicals may be either synthetic compounds intentionally added to foods or naturally occurring, biologically derived substances, i.e., *naturally occurring antimicrobials*. These substances may exhibit antimicrobial properties in the foods they normally are found in or may be used commercially as additives to other foods requiring preservation. Numerous naturally occurring antimicrobial agents are present in animal and plant tissues, where they probably evolved as part of their hosts' defense mechanisms against microbial invasion. Naturally occurring antimicrobials include those present in or derived from plant or animal tissues and those produced by microorganisms.

Recognition of the importance of food preservation has been growing as a result of urbanization, dramatic increases in world population, greater understanding of foodborne pathogenic microorganisms with the

ability to multiply even in foods stored at cold temperatures, and increased numbers of immunocompromised or immunodeficient humans, who are more susceptible to microbial foodborne illness. In addition to demanding ever greater amounts of wholesome and safe foods with long shelf-lives, consumers prefer high-quality foods that are minimally processed, palatable, convenient to use, available throughout the year, nutritious, and economical. Consumers also prefer foods that contain minimal amounts of added synthetic chemicals.

The approval and use of chemical additives, including antimicrobial agents, in food have been challenged by consumers concerned that these substances have potentially harmful effects on human health, compromise the nutritional quality of food, or mask undesirable characteristics. One task of the regulatory authorities, however, is to assure the public that no such problems exist among approved chemical agents and that, generally, any potential risks from approved antimicrobials in foods are negligible or nonexistent whereas the benefits of these antimicrobials are real and well documented. Benefits include extended product shelf-life and wholesomeness, and control of the risks and consequences of foodborne pathogenic microorganisms.

Consumer perceptions that safety problems are associated with synthetic food antimicrobials have generated interest, among members of the food industry, in the use of naturally occurring compounds with antimicrobial properties. It bears mentioning, however, that approved antimicrobial agents, such as organic acids, produced by the industry through chemical synthesis also are found naturally in many food products, and their safety as food additives is ensured by regulatory authorities. The direct isolation of these agents from natural sources can be complex, inefficient, and expensive. As indicated, however, synthetic agents are considered less desirable by a segment of the consuming public than naturally occurring antimicrobials are, and the food industry would prefer to label their products as containing only those ingredients that consumers consider "natural," as opposed to providing a long list of complex names for synthetic

chemical compounds. Thus, interest in and incentive for development, exploration, and use of naturally occurring antimicrobials in foods have increased because of the growing interest in so-called *natural* foods, and because of the potential of these antimicrobials to help preserve foods destined for consumption in developing countries with increasing needs for food and limited refrigeration and distribution systems.

The antimicrobial, chemical, and toxicological properties of a substance are influenced by a variety of factors that should be taken into account when it is considered for use as a component of a preservative system. These factors include the chemical properties of the antimicrobial and of the food to be preserved, the environmental conditions under which the food will be stored, and the types and numbers of microorganisms to be controlled.

Selection of the proper antimicrobial generally is challenging because of the complexity of foods, the variety of factors influencing preservation, and the varied chemical properties of antimicrobials. This report discusses the chemistry, occurrence, activities, action mechanisms, uses, potential for application, and research needs regarding antimicrobials in the categories of lipids, plant substances, polypeptides, and microbial metabolites. In addition, traditional and commonly used organic acid antimicrobials are discussed briefly, especially their interactions with natural antimicrobials. Traditional antimicrobials include sugar, common salt, and wood smoke. Most of the approved organic acid antimicrobials discussed, e.g., acetic, benzoic, lactic, propionic, and sorbic acids, although found naturally in various products, are synthesized chemically as additives for inclusion in food formulations but have a long history of safe use. Naturally occurring fats or oily substances from animals and plants may possess antimicrobial activity. They act as fatty acids or products of their oxidation such as peroxides, fatty acid esters, and lipopeptides. Their contribution to food preservation is difficult to estimate.

The antimicrobial agents present in plants are believed to have developed as part of their defense mechanisms against invasion. Numerous compounds exerting antimicrobial activities of variable intensities exist naturally in the barks, stems, leaves, flowers, and fruits of plants. Often these naturally occurring antimicrobials are the same compounds that in the forms of herbs and spices or essential oils have provided distinctive flavoring properties for thousands of years. Some of the most common plant products known for centuries for their antimicrobial properties belong to the genus *Allium* and include garlic, onion, and leek,

which owe their antimicrobial activity to compounds such as allicin, an inhibitor of sulfhydryl-containing enzymes. Common compounds that are present in spices and herbs and that exhibit antimicrobial activity are phenolic in nature and include eugenol and thymol. Widely-known spices with antimicrobial properties include cinnamon, clove, and allspice. Concentrations needed to provide measurable antimicrobial activity generally are higher than those needed to provide flavoring effects. In some plants, antimicrobial activity also may be present in pigments such as anthocyanins. A well-known phenolic compound with antimicrobial properties that is found in olives and olive oil is oleuropein and its derivatives. Antimicrobial activity also is present in the flowers of the hop vine used as a flavoring in beer, as well as in coffee, tea, kola, and cocoa, which contain the well-known antimicrobials caffeine, theobromine, and theophylline. Phytoalexins are broad-spectrum antimicrobial agents of low molecular weight that are produced in plants such as potato, pepper, eggplant, carrot, peanut, and grape as a response to microbial invasion. Certain beneficial microorganisms also produce secondary metabolites with antimicrobial activity against competitor microflora.

A large group of naturally occurring antimicrobial agents are polypeptidic in nature and have been isolated from plant and animal tissues, where they serve as important defense mechanisms against pathogenic invasion, or are produced by microorganisms. One type of defense based on polypeptides includes oxygen-dependent enzymes or metabolic processes yielding toxic metabolites of oxygen such as hydrogen peroxide, superoxide ions, and other radicals. Oxygen-independent defense mechanisms in plants and animals are based on peptide molecules that react and disrupt cell surfaces or membranes of microorganisms or interfere with nutrient utilization. Natural antimicrobials classified in this group include cationic proteins, lytic enzymes such as lysozyme, hydrolases such as lipases and proteases, and bacteriocins such as nisin. Other polypeptides, e.g., transferrins, act against microorganisms by binding or making unavailable certain essential nutrients such as iron ions and vitamins.

Lytic enzymes, including lysozyme, are found in many foods, including egg whites and milk. Lysozyme activity involves cleavage of glycoside bonds in the bacterial peptidoglycan, which leads to punctured cell walls and potentially to cell *lysis* (digestion). This phenomenon makes lysozyme activity particularly effective against gram-positive bacteria. Because of their limited peptidoglycan content, gram-negative

bacteria are protected by lipoprotein/lipopolysaccharide (LPS) layers and require sensitization to lysozyme by treatment with chelators such as ethylenediaminetetraacetic acid (EDTA). Other enzymes able to cleave glycosidic or peptide linkages in the cell walls of bacteria and fungi include glycosidases, amidases, endopeptidases, and lipases. Their potential as antimicrobials in food need to be evaluated. Bacteriolytic enzymes also are produced by microorganisms; however, their potential—either alone or in mixtures—for use in food preservation, and the safety of their use also need further evaluation.

Other enzymes with antimicrobial activity include plant and bacterial chitinases, peroxidases, and oxidases including lactoperoxidase, which is found in bovine milk. By binding hydrogen peroxide and catalyzing oxidation of electron donors such as thiocyanate, lactoperoxidase creates intermediate oxidation products that act as antimicrobials. The lactoperoxidase antimicrobial system, i.e., lactoperoxidase/thiocyanate/hydrogen peroxide, yields oxidation products that act on microbial cell membranes and interfere with metabolism of cells and division of microorganisms such as *Listeria monocytogenes*, *Salmonella* serovars, and *Campylobacter* species. Antimicrobial peroxidase enzymes other than lactoperoxidase also are present in mammalian tissues and secretions and include myeloperoxidase; antimicrobial effects also can develop from the activity of glucose oxidase coupled to other oxidases. Natural antimicrobial activity has been associated with iron ion-binding peptides such as transferrins, e.g., ovotransferrin or conalbumin, and lactoferrin. These peptides are present in products such as eggs and milk and exert an antimicrobial effect by depriving microbial cells of iron ions.

Several classes of small, naturally occurring peptides with an antimicrobial effect could be useful in the control of undesirable microorganisms in foods. Included are bacteriocins, defensins, killer toxins, and lipopeptides. *Bacteriocins*, antibacterial peptides produced by microorganisms, were discovered in 1925 in strains of *Escherichia coli*. It now is known that bacteriocins are produced by many genera of microorganisms, including *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* used in lactic acid food fermentations. Although they act as potential inhibitors or cidal agents against sensitive microorganisms under certain conditions, bacteriocins in foods may cause moderate antimicrobial activity followed by microbial growth, which may indicate development of resistance, application of inadequate quantities of bacteriocin, or its inability to find all cell microenvironments to inactivate the target microorganism. The

potential for commercial use of bacteriocins may be enhanced when they are used in multihurdle preservation systems. Bacteriocins produced by lactic acid bacteria include nisin, lactococcins, lactacins, lactacins, diplococcin, sakacins, acidophilocins, pediocins, and leuconosins. As an inhibitor of spore-forming *Clostridium* spp., which cause cheese blowing due to undesirable gas production, nisin was the first bacteriocin produced by lactic acid bacteria to be isolated and approved for use in cheese spreads. Although mostly active against gram-positive bacteria, bacteriocins can be cidal under certain conditions, even towards gram-negative bacteria and yeasts, provided that their cell walls have been sensitized to their action. The antimicrobial action of nisin and of similar bacteriocins is believed to involve cell membrane depolarization leading to leakage of cellular components and to loss of electrical potential across the membrane.

Killer toxins are peptides produced by strains of yeasts, e.g., *Saccharomyces cerevisiae*, that inactivate sensitive strains of related yeasts. Their presence in grape musts may contribute to sluggish or stuck wine fermentations. *Defensins* are a family of small molecular weight cationic peptides that inactivate microorganisms by forming pores on cell membranes and by increasing ion permeability. Their potential for use as natural antimicrobial agents in foods is not known. Aiding in an insect's defense against microorganisms are chemical antimicrobials such as short-chain fatty acids, caffeic acid, and quinones. Insects also are known for their inducible immune system, which is based on lysozyme and the secretion of antimicrobial peptides in the haemolymph. Antimicrobial peptides found in insects include the defensins, cecropins and attacins, which may act synergistically with lysozyme. The antimicrobial activity of these peptides depends on peptide type and bacterial species. Insects also are a source of other unidentified chemical antimicrobials as well as of antimicrobial compounds derived from potentially survival-enhancing microorganisms found in association with insects. Some of these substances have been investigated and even patented as antimicrobial agents. Although not explored, the use of insects as sources of antimicrobial food preservatives may become a reality; the honey bee, for instance, introduces into honey the antimicrobials peptide royalecin and hydroxydecanoic acid.

Natural antimicrobials that bind vitamins essential for microbial growth are present in bovine milk and in egg albumen, in the form of proteins such as avidin. Food proteins with antimicrobial activity also include caseins, which act by binding water and de-

creasing water activity or by forming derivatives with antimicrobial activity. Antibacterial activity also is present in inhibitors of proteinase activity, such as aprotinin, protamine, and histones, which may be useful in sensitizing bacteria to other antimicrobial agents.

Antimicrobials produced by microorganisms are not limited to organic acids and bacteriocins but include compounds such as ethanol, natamycin, diacetyl, and hydrogen peroxide produced by yeasts or lactic acid bacteria. Not only are these compounds well-known natural antimicrobials, but their use in certain foods has been approved or stated in the U.S. Code of Federal Regulations. Ethanol produced through alcoholic fermentation of sugars by yeasts and in small amounts by heterofermentative lactic acid bacteria is effective generally at high concentrations, and its use as an intentional food preservative is limited. Natamycin is a polyene macrolide antibiotic active against yeasts and molds but ineffective against bacteria; its use is approved in several countries. Hydrogen peroxide has been used as a well-known antimicrobial since its discovery in 1818 and is approved for use as a direct antimicrobial in dairy products and for the sterilization of packaging materials and other surfaces coming into contact with food. *Diacetyl*, the buttery flavor in certain dairy products, is produced by the citrate fermenting group of heterofermentative lactic acid bacteria and has antimicrobial properties. Amounts needed for microbial inhibition, however, generally are greater than those needed for acceptable buttery flavor.

Food preservation can be assisted not only by naturally occurring chemical agents with individual antimicrobial effects but also by interactions among multiple antimicrobial factors resulting in additive or synergistic effects. These factors include natural product composition, microbial flora, acidity, water activity, processing and storage temperatures, added chemicals, and packaging. This approach to food preservation, known as the *hurdle* or *barrier* concept or principle, is based on the application of multiple factors that act as hurdles resulting in the inactivation or the extended inhibition of undesirable microbial growth. According to this concept, the intensity or concentration of a single antimicrobial agent or factor is inadequate to achieve preservation. But because a combination of factors are applied or employed at levels less than optimal for the target microorganism, their additive, or synergistic, effects can prevent or delay undesirable microbial activity. This approach (hurdle) is very useful in food preservation because factors or antimicrobials should be applied at levels

or concentrations having no adverse effects on product quality and safety. Furthermore, the activity of naturally occurring antimicrobials may be enhanced or decreased by storage temperature, water activity, acidity, and processing of foods, and their use at concentrations needed for antimicrobial activity may be limited by their flavor or aroma intensity.

For application of a naturally occurring antimicrobial to a food there is a need to determine its efficacy in vitro and in food products, and the antimicrobial spectrum of the compound should be as broad as possible. Antimicrobial activity often depends on the type, genus, species or strain of microorganism, and number of microorganisms present. The antimicrobial selected 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. Important intrinsic and extrinsic factors or variables associated with application of an antimicrobial to a food that should be evaluated during in vitro testing include temperature, atmosphere, pH oxidation-reduction potential, and water activity.

To be useful as a natural food antimicrobial, a compound must function in a food system. A number of natural antimicrobials exist that could be used immediately in foods, but few actually have been utilized—mainly because their effectiveness in foods has not been investigated thoroughly.

Application testing can be very complex and include a number of the variables mentioned above, which can be evaluated in model systems or in actual foods. Many antimicrobials act together and therefore might be most appropriately evaluated in combination with decreased levels of approved antimicrobials. Success of application testing may be determined by increased shelf life or decreased potential health hazards.

The exact mechanisms through which antimicrobials affect microbial growth are complex and difficult to elucidate, but the best method for determining the most useful antimicrobial type may be based on its mechanism of action and/or its target in the cell. Mechanisms of action of food antimicrobials may include reaction with the cell membrane causing permeability changes or interference with uptake and transport functions, inactivation of essential enzymes, interference with genetic mechanisms, or inhibition of protein synthesis. Knowledge of the antimicrobial mechanism of a compound may allow selection of combinations of antimicrobials with different mechanisms that could be utilized against the microorganisms in a food product.

The chemical properties of an antimicrobial com-

pound must be examined, as well as its purity for the purpose of establishing specifications and for regulatory approval. Related is the need for development of an assay method for a compound to determine input levels and stability. In addition, the most sensible method of commercial application should be selected.

Perhaps the most important aspect of any compound proposed for use as a food preservative would be its toxicological properties. Because they occur in nature, naturally occurring antimicrobials often are thought to be less toxic than synthetic compounds, but this is not always true. A naturally occurring antimicrobial must be demonstrated to be nontoxic either by animal testing or by its continuous consumption by consumers as a food over a long period. In addition to lacking toxicity, they must be nonallergenic and be able to be metabolized and excreted so as to not lead to residue build-up. Food antimicrobials should not react either to make important nutrients unavailable to humans or to destroy these nutrients.

Food additives in general and preservatives in particular are regulated in the United States by agencies dealing with food products, e.g., the Food and Drug Administration (FDA) and the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS). As indicated, one of the supposed desirable properties of naturally occurring antimicrobials is their decreased impact on the labeling of foods. Consumers reportedly are concerned about the presence of synthetic chemicals in their foods and would prefer natural compounds. A potential problem with natural antimicrobials might be that in the highly purified form they would need to be approved as food additives for use as food preservatives. This would involve expensive and time-consuming toxicological testing. In addition, they likely would have to be labeled as chemical additives. Of course labeling in this manner would defeat the purpose of using a natural compound. Thus, less purification probably is 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 approval for use. This is possible only if the product from which the extract is taken is known to be nontoxic.

Another major factor needing to be addressed before applying naturally occurring antimicrobials is their potential impact on the sensory characteristics of a food. Many naturally occurring antimicrobials must be used at high concentrations to achieve antimicrobial activity against microorganisms. Obviously, compounds that negatively affect flavor and odor or contribute inappropriate flavors and odors would be unacceptable. For example, many spice extracts

have antimicrobial activity but, at the concentration required for antimicrobial activity, would cause a food to be inedible to most consumers. In addition to adverse effects on flavor, odor, or texture, it would be unacceptable for a food antimicrobial to mask spoilage, as this could protect consumers from ingesting foodborne pathogens.

Perhaps the greatest roadblock to the use of naturally occurring antimicrobials could be economics. For example, the only antimicrobial enzymes currently produced at a cost to be useful in food preservation are lysozyme and glucose oxidase. A potential antimicrobial must pay for itself by extending shelf life and/or minimizing the chances of foodborne illness. Depending on the perishability of a food product, even an additional 2 to 3 days of shelf life can offset the cost of an antimicrobial significantly.

A naturally occurring antimicrobial would be ideal if effective enough to be added as a whole food or as an edible component, e.g., an herb or a spice. Few, if any, antimicrobials are present in foods at concentrations great enough to be antimicrobials without purification or concentration. Often, even if purification of antimicrobials is possible, their addition to another food may lead to undesirable sensory changes. The ultimate challenge is to find a naturally occurring antimicrobial that can be added to a "microbiologically-sensitive" food product in a nonpurified form from another nonsensitive food. The nonpurified food would have to contain an antimicrobial that is completely nontoxic and highly effective in controlling the growth of microorganisms.

As has been indicated, consumers demand minimal processing and limited use of synthetic chemicals in foods while preferring products that offer high quality, long shelf-life, and convenience, as well as safety. These demands should encourage exploitation of naturally occurring antimicrobials. Of the many natural antimicrobial systems or components discussed, however, only a few have been exploited, tested, or applied. For more extensive use of natural antimicrobial systems, there is a need for research to examine the extraction, isolation, safety, efficacy, and interaction with food components and other preservative systems, action mechanisms, application methods, and product quality influences, e.g., nutritional and sensory. In addition, care should be taken to ensure that any natural antimicrobial system applied on a commercial scale is safe, addresses a real need, and consists of readily available and economical components. Furthermore, it should be realized that certain natural antimicrobial systems may be appropriate only for certain populations, in which they can aid efforts to

address world food supply problems. Even then, however, social/ethical concerns should be taken into account before an application is endorsed.

Naturally occurring antimicrobial agents are abundant in the environment. They are present in plant and animal systems or produced by microorganisms. Their antimicrobial activity has been demonstrated in model as well as in actual food systems, and some naturally occurring antimicrobials have found commercial application in food preservation. The desire for expanded use is obvious, especially in light of consumer demands for minimally processed safe foods of

adequate shelf life and convenience and the global need for an increasing supply of food. For the use of naturally occurring antimicrobials to increase, commercial development and economic production must occur on an adequate scale, sensory changes in foods must be avoided, food safety/toxicity must be assessed, multifactor preservative systems including natural antimicrobials must be developed, and technology transfer must occur. With economical preservation systems based on natural antimicrobials, the world will have an important weapon in the struggle against hunger.

1 Introduction

Food is one of the necessities of human existence in that it supplies the energy for the chemical reactions, permitting bodily function, as well as the chemicals, needed for growth, reproduction, maintenance, and repair of damaged or older cells. Food consumption also provides occasion for pleasurable social experiences (Banwart, 1989). Food products deteriorate and decay, however, as a result of microbial growth and chemical and enzymatic reactions (Foegeding and Busta, 1991; Sofos and Busta, 1992). Changes occurring during product storage include those in general appearance, color, flavor, texture, consistency, nutritive value, and/or toxicity. Generally, microbial growth in food leads to the product's becoming unfit for human consumption. Thus, microbial growth contributes to worldwide food shortages, with all their ethically and economically undesirable consequences.

Food losses and associated adverse effects caused by undesirable microbial growth can be decreased or prevented through the application of food preservation methods extending product shelf-life, retaining wholesomeness and nutritive value, preventing product decomposition, and ensuring safety by suppressing the growth of pathogenic microorganisms. Over thousands of years, what are now common methods of food preservation, e.g., drying or dehydrating, fermenting, heating, salting, and exposing to wood smoke, were developed through chance, observation, and trial and error. Application of these methods allowed humans to preserve foods that could be eaten during periods of crop failure, inadequate game, adverse weather, and migration. With time and in light of an increasing human population, food preservation gained importance and objectives were expanded to include decreased food waste, maintenance of product wholesomeness, and assurance of public health and safety.

Expansion and advances in processing and distributing foods, development of "fresh," yet relatively perishable food products, increased acceptance and use of convenience foods, centralization of food processing operations, decreased numbers of people involved in food production, increased world population, and

changes in lifestyles have increased the potential for mishandling of foods during various stages of processing, storing, distributing, retailing, and preparing. In addition, there is a trend towards production of high-quality food products with a long shelf-life but with minimal processing and no preservatives. Other modern developments are increased life expectancy and increasing proportion of immunocompromised individuals, who are more susceptible to illnesses caused by microorganisms that may proliferate in foods. For these reasons, food preservation is becoming ever more critical to the survival and well-being of humans (Sofos and Busta, 1992). This trend has led to the improvement of existing and to the development of new food preservation systems.

Important systems of food preservation include handling aseptically to prevent or to minimize entry of microorganisms into the food; removing microorganisms through washing, centrifugation or filtration; destroying microorganisms with heat, gases, or irradiation; and/or inhibiting microorganism growth through control of the environment, addition of chemical food preservatives, or competition by desirable microorganisms (Table 1.1).

Approved and widely used physical methods of food preservation include drying or dehydrating, refrigerating or freezing, and pasteurizing or canning. Irradiation treatments also are considered physical, but their commercial application even for approved uses in food preservation in the United States is limited because of potential or perceived consumer concerns, labeling requirements, technologic and economic considerations, and product quality (Council for Agricultural Science and Technology, 1996).

Chemicals added or applied as antimicrobials to foods or to food processing/handling environments include acids, esters, alcohols, aldehydes, metals, halogens, phenols, cresols, quaternary ammonium compounds, biguanides, antibiotics, dyes, metal chelate complexes, organic arsenic compounds, organic mercury compounds, silver compounds, and synthetic antibacterial, antiviral, and antifungal agents (Russell, 1991). Some of these chemicals are used as disinfectants, sterilants, or antiseptics; others are used

as preservatives of foods, cosmetics, or pharmaceuticals. The effectiveness of these chemicals against microorganisms depends on the type of organism and environment, the properties of the chemical agent, the intensity of its dose, and the duration of exposure.

Chemical agents are especially useful in products in which use of physical methods of preservation may have adverse effects on product quality and acceptability. A *food preservative* can be defined as any agent, though it generally is chemical, that when applied to food extends shelf-life and maintains quality and safety by retarding or preventing microbial growth and deteriorative changes affecting appearance, flavor, odor, color, texture, and/or nutritive value. According to one definition, a *chemical antimicrobial food preservative* is an agent inhibiting or inactivating undesirable microorganisms. Chemical antimicrobials may be used alone, in mixtures, or in combination with physical methods of food processing and preservation. Use of combinations allows the use of lower individual doses, which have fewer undesirable effects than higher doses do on quality and other properties of the original product (Foegeding and Busta, 1991; Giese, 1994; Sofos and Busta, 1992). Chemical antimicrobial food preservatives commonly used in various countries appear in Table 1.2.

All known antimicrobial agents have disadvantages when used in food preservation; none is ideal for use in every food product. An effective antimicrobial should be able to inhibit all types of undesirable microorganisms that may be present in the product to be preserved. The antimicrobial would be especially desirable if it did not interfere with the proliferation of desirable microorganisms such as lactic acid-producing bacteria in a product such as fermented foods, where the activity of such microorganisms is needed. Use of an antimicrobial in a product should not lead to the development of resistant microbial strains, which is one reason that medically important antibiotics have not been approved for use as food preservatives. The chemical and physical properties of the antimicrobial agent should be compatible with the composition and properties of the food to be preserved. Important properties to be considered include chemical reactivity, solubility, dissociation constant (pKa), toxicity, and influence on product quality (Foegeding and Busta, 1991; Lück, 1992; Sofos and Busta, 1992).

The application of chemical antimicrobial agents in food preservation is regulated in the United States by the Food and Drug Administration (FDA) of the Department of Health and Human Services and in other countries by appropriate corresponding authorities (Ahlborg et al., 1977; Foegeding and Busta, 1991;

Owen Fields, 1996; Post, 1996; Sofos and Busta, 1992). Chemical antimicrobials in the United States are regulated according to the Food Additives Amendment of the Food, Drug and Cosmetic Act, which is administered by the FDA. The act and its amendments specify the procedures and conditions required for a chemical food additive to be approved. At the international level, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations examine usage and safety of chemicals in food and recommend acceptable daily intakes (ADI).

Before its approval, a compound must be evaluated and its safety established through experimentation in animals and possibly in human clinical trials. Regulations describing use specify approved applications, amounts to be used, and conditions necessary to protect public health. Approved additives can be found in the Code of Federal Regulations, Title 21, Part 182 (Parts 180 through 189 and other parts pertinent to the use of chemical preservatives). Certain chemical additives, including antimicrobials such as acetic acid, sorbic acid, and sulfur dioxide have been classified as generally recognized as safe (GRAS) in the United States and are exempted from food additive regulations. These GRAS substances, however, may be used only in approved products and within approved conditions of use, according to good manufacturing practices (GMP). Addition of a chemical to a food must be declared on the product label (Sofos and Busta, 1992).

Approval and use of chemical additives in foods have been questioned because of concerns relating to potentially harmful effects on human health; decreased nutritional quality of food; masking of undesirable characteristics in unwholesome products; and compromised or decreased attention to the application of GMP (Foegeding and Busta, 1991; Parke and Lewis, 1992). These potential problems generally are addressed by the regulatory authorities of each country before a chemical is approved for use in foods or during inspection of product manufacture or preparation. Conditions to be evaluated before a chemical is approved (Foegeding and Busta, 1991; Sofos and Busta, 1992) are sevenfold.

1. There should be an obvious or justifiable need for preservation.
2. The efficacy of the proposed additive in fulfilling that need should be proven.
3. The proposed compound should be proved nontoxic and noncarcinogenic at concentrations exceeding those of the proposed use.
4. Addition of the proposed compound should not al-

ter appearance, flavor, or nutritional quality of the food.

5. Application of the proposed compound should be feasible and compatible with its chemical properties, e.g., solubility or pKa.
6. The proposed compound should be affordable and available in quantities adequate to fulfill projected needs.
7. The total projected consumption of the proposed compound, including consumption from all other sources, should be below the ADI, i.e., the level considered safe in terms of human health.

These strict requirements have made it difficult to obtain approval for new chemicals for use as antimicrobials in food. Most of the currently used chemical antimicrobials were approved in the past and had been proven safe through long, sometimes accidental, use. In addition, negative publicity associated with the use of chemical additives in food processing has made processors reluctant to use chemicals that they would be required to declare on product labels. On the other hand, the food processing industry would be receptive to the use and declaration of substances classifiable as *natural*, because consumers would be more receptive to buying products whose labels bear a list of such ingredients. Natural antimicrobial agents, however, also must meet the above requirements before being approved for addition to foods.

Table 1.1. Classification of food preservation methods (modified from Dillon and Board, 1994a; Gould, 1992, 1996)

Action on microorganisms	Method of preservation
Denial of access in germ-free foods	Aseptic handling or packaging
Removal of microorganisms and reduction of contamination	Washing Filtration Centrifugation
Inactivation of microorganisms	Pasteurization/appertization/canning (heating) Ionizing radiation Microwaves Application of hydrostatic pressure Electric/Light pulses Electroheating Ultrasonics
Inhibition of growth	Refrigeration/freezing (reduced temperature) Drying/dehydration/salting/sugaring (reduced water activity) Acidification (increased acidity) Modified atmospheres (vacuum, gases) Addition of chemicals/antimicrobials
Promotion of growth and production of antimicrobials	Fermentation (microbial activity)

Table 1.2. Common chemical antimicrobials used in foods in various countries (from Tranter, 1994)

Antimicrobial	Preserved foods	Sensitive organisms
Propionic acid/propionates	Bread, cakes	Molds
Sorbic acid/sorbates	Hard cheeses, syrups, cakes, salad dressings	Molds, yeasts, some bacteria
Benzoic acid/benzoates	Margarine, relishes, soft drinks, salad dressings	Yeasts and molds
Sulfur dioxide/sulfites	Molasses, dried fruits, wines, some meat products	Bacteria and some yeasts
Sodium diacetate	Bread	Molds
Nisin	Cheese spreads, canned foods	Lactic acid bacteria, clostridia
Ethyl formate	Dried fruit, nuts	Yeasts and molds
Sodium nitrate/nitrite	Meat products	Bacteria
Sodium chloride	Meat products, fish	Bacteria
Citrate	Soft drinks	Bacteria
Acetate	Pickled vegetables	Bacteria
Nystatin	Bananas	Molds
Chlortetracycline	Fish	Bacteria
Ethylene/propylene oxide	Nuts and spices (fumigants)	Yeasts and molds

2 Role and Need for Antimicrobials in Food

A very important challenge facing humans today is the ever-increasing world population. Although the birthrate has been decreasing in recent years, the death rate also has been declining, and world population overall has continued to increase. Demographers indicate that in the 230 years between A.D. 1600 and 1830, world population doubled, from half a billion to one billion people; world population then doubled again by A.D. 1930, reaching two billion people. If current trends continue, the population of five billion current in the 1990s will double again within approximately 40 years (Potter, 1986).

While world population increases rapidly, predictions regarding future food supplies are pessimistic. The localized and seasonal famines of the 1980s and 1990s are expected to become more commonplace. Some researchers estimate that, even in the 1980s, 12.5% of the world's population received less food than necessary, and as many as half the people on earth received marginal amounts of food (Banwart, 1989). Optimists predict that food supplies will be adequate to feed extensive numbers of people. Most experts agree, however, that the rate of population increase needs to decrease substantially if food supplies are to be adequate. Additionally, to avoid future famines, preservation and reservation of food supplies are necessary. Scientists continue to seek ways to increase food production and to prevent loss of existing foods.

Food losses are due to, in addition to climate, the activity of microorganisms, nematodes, insects, rodents, birds, and other pests. Food products also may become unacceptable for consumption through the actions of inherent enzymes or of enzymes produced by microorganisms growing on the product. As organisms grow and chemical reactions take place, complex carbohydrates or polysaccharides are degraded and form lower molecular weight sugars such as oligosaccharides, disaccharides, and monosaccharides, which are metabolized to yield organic acids, e.g., lactic, acetic, and formic acids, as well as ethanol, carbon dioxide, and water. Hydrolysis of lipids results in formation of glycerol and free fatty acids, whereas proteins are broken down to yield polypeptides, oligopeptides, and free amino acids, which result ultimately in the

release of odorous ammonia, hydrogen sulfide, and amines such as indole and skatole (Shapton and Shapton, 1991). Food spoilage of this type may be initiated or enhanced by insect damage or physical injury, including bruising and dehydration.

Information on actual food waste or losses due to decay, damage, deterioration, and spoilage is difficult to gather and thus unreliable, but no doubt losses are high, for they can occur at every step of the food chain, from production to consumption. Estimates of food losses depend on the source of information. Some indicate that 30% of crops worldwide are lost to pests (Ennis et al., 1975); in the United States, it is estimated that 25% of all crops are lost to that cause (Albrecht, 1975). Estimates indicate that 20% of all fruits and vegetables harvested for human consumption are lost due to microbial spoilage (Jay, 1992) (Figures 2.1 and 2.2). A study released by the U.S. Department of Agriculture (USDA) on July 1, 1997 indicated that more than one-fourth of all the food produced in the United States is wasted (www.econ.ag.gov/whatsnew/feature). Total annual monetary losses from both pro-



Figure 2.1. *Trichoderma*, a fungal contaminant, on an orange. Photograph reproduced from Snowden, 1995a.

ducers and consumers are in the range of tens of billions of dollars (Banwart, 1989). Prevention or reduction of such extensive food losses would result in an increased food supply, which is needed to feed the earth's ever increasing population.

Of the various agents that attack food, microorganisms are of unique importance because their proliferation may result in undesirable as well as useful activities. Useful microorganisms are involved in making fermented foods such as cheeses, some bakery products, and pickles. Undesirable microorganisms are those leading to food losses through spoilage or to the development of foodborne illness, either directly or through production of microbial toxins. Microbial food spoilage results in economic losses from spoiled food, and foodborne diseases result in economic losses from morbidity and mortality (Bryan, 1992; Council for Agricultural Science and Technology, 1994).

Information compiled and presented by the U.S. Centers for Disease Control and Prevention (CDC) indicates that microorganisms are the most important of all food hazards, which include natural toxins, pesticides, chemical additives, environmental contaminants, and malnutrition. In excess of 60% of the yearly outbreaks of foodborne illness investigated are caused by bacterial contaminants. The estimated economic losses associated with these illnesses are associated with medical expenses; productivity losses for patients and families; costs to the food's processor, supplier, and preparer; illness investigation costs; and legal

costs. Estimated costs per case range from \$1,000 to \$50,000, depending on the pathogen involved (Council for Agricultural Science and Technology, 1994; Garthright et al., 1988; Roberts, 1989; Todd, 1985a, 1985b, 1989). Costs can be as low as zero or much higher than these estimates if one considers that some illnesses are very mild and require no medical attention, whereas others result in death. Overall, however, the costs to the national economy may reach billions of dollars per year (Bryan, 1992; Council for Agricultural Science and Technology, 1994), excluding loss of human life.

The problem of foodborne illness has become increasingly significant in recent years as new or emerging pathogenic, often *psychrotrophic*, bacteria, i.e., bacteria able to grow at refrigeration temperature, are being recognized and implicated as causes of foodborne illness. This concern only can become more severe as the population ages, for immunocompromised or immunodeficient human populations generally are more susceptible to foodborne illness. The obligation of scientists, regulatory authorities, and others to explore the avenues leading to avoidance of foodborne illness is clear, and the U.S. government is supporting efforts such as the National Food Safety Initiative to enhance the safety of our food supply.

In the past, people obtained much of their food through their own labor in the field or the garden. Today, with most people living in large urban metropolitan areas and relatively few involved in agriculture, much of the food consumed is processed so that when it reaches consumers it is in a state that is wholesome, nutritious, palatable, safe, and economical. In recent years, the food processing industry has become very large and complex in its attempt to provide a variety of new and modified products to the marketplace in order to satisfy changing consumption patterns. Development of new products requires advances in science and technology that will ensure among other things the wholesomeness, sanitary quality, shelf-life, and safety of new products (Troller, 1993).

As the population in large urban communities increases and the food processing and distribution systems become centralized, the need for proper food preservation grows. Consumers expect to have access to foods that are pure, wholesome, and safe; the U.S. food supply generally is considered safe. Increased handling, processing, long-term storing, retailing, and distributing increase the risk of spoilage and contamination by pathogens, however. In addition, new technologies in food production, processing, or distribution may introduce hazards or magnify existing ones and



Figure 2.2. A melon infected with several species of fungi. Photograph reproduced from Snowden, 1995b.

thus increase risk to consumers.

Antimicrobials present in or added to foods may be classified as synthetic, naturally occurring, or biologically derived, which also may be considered natural (Davidson and Branen, 1993). As direct additives to food, antimicrobials are of importance to the food industry because they maintain freshness and control pathogenic bacteria. Other food additives include those used to improve or to maintain nutritional quality, to aid in processing, and to maintain or to improve sensory characteristics. As indicated, introduction of new marketing techniques such as the vacuum packaging of fresh, raw, and cooked refrigerated foods has increased interest in antimicrobials (Davidson and Branen, 1993; Dillon and Board, 1994a; Jay, 1992). Development of convenience foods, low-calorie products, and dietetic substitutes also has necessitated the use of additives and antimicrobials. In this sense, today's food marketing system requires the use of chemical additives in the food supply (Branen, 1993; Dillon and Board, 1994b; Gould, 1992).

Whereas the need for food antimicrobials may be widely recognized, their safety has been disputed, and this has led the FDA to approve no new food antimicrobial in recent years. Although the safety of processed foods and chemical additives has been questioned, public health problems due to pathogenic microorganisms, especially for the young, the elderly, and the infirm should be recognized and the contribution of preservatives to the avoidance of such problems should be appreciated. Any potential risks from inclusion of antimicrobials in foods should be

negligible and should be balanced against the benefits derived from such inclusion, e.g., reduction of food losses and avoidance of microbial foodborne illness.

Because of potential or perceived toxicological problems associated with synthetic food antimicrobials, the food industry has become increasingly interested in naturally derived compounds with antimicrobial properties (Stroh, 1994). It is well-known that countless chemicals in nature have the ability to affect microorganisms. As indicated, these are recognized as natural antimicrobial agents (Beuchat and Golden, 1989; Conner, 1993; Dillon and Board, 1994c; Gould, 1992, 1995, 1996; Jay, 1992; Marth, 1966; Shelef, 1984; Wilkins and Board, 1989). Naturally occurring antimicrobial agents (Table 2.1) are present in animal or plant tissues or are produced by microorganisms (Dillon and Board, 1994c). In their defense against microbial attack and infection, animals and plants have developed defense mechanisms present in the tissues, as components of mucosal secretions, or in the blood and the lymphoid tissues (Ekstrand, 1994). Examples of such defense factors include lysozyme, lactoperoxidase, and lactoferrin/ovotransferrin in milk and eggs (Reiter, 1986). Various attempts have been made to study these natural defense agents for their potential applications as naturally occurring food preservatives. This report provides an overview of naturally occurring antimicrobials by summarizing available information dealing with their occurrence and chemistry, antimicrobial activity and mechanism of action, and uses or potential for applications in foods.

Table 2.1. Major natural antimicrobial systems and potential for synergy (from Gould, 1996)

Origin	Example	Antimicrobial
Animals-constitutive	Phagosomes	Myeloperoxidase
	Serum	Transferrins
	Milk	Lactoperoxidase, lactoferrin
	Eggs	Lysozyme, ovotransferrin, avidin
Animals-inducible	Immune system	Antibodies, complement
	Frogs	Magainins
	Insects	Attacins, cecropins
Plants-constitutive	Herbs, spices, and other plants	Eugenol (cloves), allicin (garlic), allyl isothiocyanate (mustard)
Plants-inducible	Injured or infected plants	Low MW ^a phytoalexins, high MW polyphenolics
Microorganisms	Lactic acid bacteria	Nisin, pediocin, other bacteriocins
	Other microorganisms	Other antibiotics (pimaricin, subtilin), bacteriophages, yeast "killer toxins," organic acids, and other low-MW metabolites
Potential synergists	Low pH	Organic acids
	Low water activity, chelators	Specific solutes
	Low oxygen,	
	increased carbon dioxide	Raised carbon dioxide
	Mild heat, high pressure	Low temperature

^aMW = molecular weight

3 Description of Antimicrobials

Introduction

The oldest and most traditional antimicrobial employed in food preservation is common salt, or sodium chloride, which has been used in meat and other foods for centuries. The first use of salt, as well as the application of other traditional methods of food preservation, likely was accidental. Its usefulness was confirmed and its use continued through observation and trial and error. Some preservative methods probably were used initially to improve sensory properties of food (Foegeding and Busta, 1991; Sofos, 1984).

In addition to salt, other preservatives such as acids were discovered or developed, mostly during this century, and the main objective for their use in foods is inhibition of microbial growth. Certain food additives, e.g., antioxidants and phosphates, used in processed foods for objectives other than antimicrobial activity also have been found to have an inhibitory effect against microorganisms (Davidson and Branen, 1981; Shelef and Seiter, 1993). This effect may be due to a synergistic action with other antimicrobials or through modification of food properties. Such additives are considered to have indirect antimicrobial effects.

Many of the synthetically produced compounds approved and used as additive antimicrobials also are present as natural components of certain foods. Chemicals with antimicrobial activity that are present in the food supply therefore may be classified as traditional additives of direct or indirect activity, and as naturally occurring compounds intentionally added or naturally present. Direct antimicrobial food additives approved for use in foods include organic acids and their derivatives, esters, gaseous preservatives, nitrates and nitrites, and miscellaneous antimicrobials; only those substances with natural links are discussed in this report. *Indirect antimicrobial agents*, that is, chemicals added to achieve other primary objectives but also potentially contributing antimicrobial activity, i.e., phosphates, phenolic antioxidants, and ethylenediamine tetraacetic acid (EDTA) (Davidson and Branen, 1993; Sofos and Busta, 1992), are not discussed in this report.

None of the currently commercially available and approved antimicrobials is ideal, or capable of fulfilling all the needs of microbial inhibition in food preservation. This is not surprising, because of the wide variety of microbial agents and foods needing to be preserved with no harm to human health. Antimicrobial activity is influenced by a variety of factors associated with properties of the agent and the food and with storage conditions (Davidson and Branen, 1993; Foegeding and Busta, 1991; Giese, 1994; Lück, 1992; Sofos and Busta, 1992; Welbourn, 1994). The activity and interaction of these factors should be monitored and taken into account when new processed food products are developed. Antimicrobial activity depends on the compound, microorganism, and food to be preserved.

The antimicrobial spectrum of a compound should be as wide as possible, and inhibition of one microorganism should not modify the chemical, environmental, or microbiological balance of the food in such a way that growth of other potentially pathogenic species is allowed or food becomes unsafe for human consumption. In such an instance, inhibition of the first by an antimicrobial could result in growth of a second microorganism, resulting in unexpected or previously unencountered problems (Foegeding and Busta, 1991).

In addition, acceptable antimicrobials should not contribute to the development of resistant strains. For example, resistance by molds present in cheeses may develop against sorbate and benzoate as a result of long-term exposure to subinhibitory concentrations (Sofos, 1989b). This concern has been the major reason that medically important antibiotics have not been allowed for routine use in food preservation (Foegeding and Busta, 1991). In instances such as those associated with products involving lactic acid fermentation, the chosen antimicrobial, e.g., sorbic acid, should suppress unwanted species without interfering with fermentation (Sofos and Busta, 1992).

In selection and approval of antimicrobials for use in food products, physical and chemical properties such as chemical reactivity, solubility, pKa value, and toxicity are very important. Esters of *p*-hydroxyben-

zoic acid, for instance, may be preferred in a high pH product, where they are active, whereas benzoic acid may be the antimicrobial of choice in lower pH formulations. Weak acid preservatives such as sorbic and benzoic acids are more effective antimicrobials at pH values approaching their pKa value because most of the inhibitory effect of these antimicrobials is due to their undissociated forms, which increase in concentration as pH decreases and approaches pKa. The pKa values of most commonly used weak acid preservatives are below pH 5.0. The salts of benzoic and sorbic acids are more soluble in water than acids are and therefore are preferred when concentrated solutions for product spraying are necessary (Sofos, 1989b). Acids and salts are especially effective when applied directly as ingredients of product formulation. The antimicrobial activity of esters of *p*-hydroxybenzoic acid increases with chain length, but solubility decreases. Therefore, a mixture of two or more compounds may be optimal for acceptable solubility and adequate antimicrobial activity (Foegeding and Busta, 1991; Sofos and Busta, 1992).

The most commonly used direct antimicrobial agents in commercial foods are weak acids and their salts or esters, most of which originate in nature. Acids are important in the preservation of pickled, acidified, and fermented foods such as cucumber pickles (Figure 3.1), yogurt, sauerkraut, and fermented sausages. Natural or direct food acidification has been used as a method of food preservation for thousands of years. In addition to direct antimicrobial activity, acids may enhance food preservation through their interaction with other processes or components of food

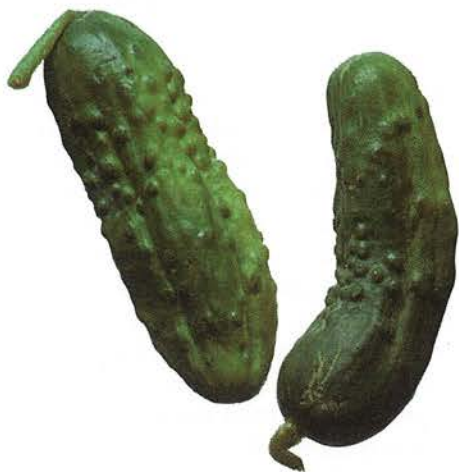


Figure 3.1. Acids are important in the preservation of pickled, acidified, and fermented foods such as cucumber pickles.

products, such as dehydrating, heat processing, and adding chemicals. Reduced pH caused by increased acidity also potentiates the antimicrobial activity of preservatives such as nitrite and sulfite and enhances microbial destruction by heat (Sofos and Busta, 1992).

When an antimicrobial agent is being selected, potential interactions with the foods to be preserved need to be considered in light of its properties. For example, preservation of a heated product may be possible with smaller amounts of preservative, because of injury to microorganisms. Many food products are maintained as stable and safe through the combined or synergistic activity of several factors contributing to antimicrobial activity. Factors may be intrinsic to the food such as natural composition, acidity, and water activity. Processing temperature, added chemicals, temperature, and gas atmosphere during storage are extrinsic. This concept of multiple factors, often termed the *hurdle* or *barrier* concept (Leistner, 1985, 1995; Leistner and Rodel, 1976; Sofos, 1993), is likely to remain an important concept in food preservation (Wagner and Moberg, 1989). Use of combinations of additives and processes may provide unlimited alternative commercial food preservation systems that would meet consumer needs for wholesome and safe foods.

Processors may introduce antimicrobials to food by adding them directly to the formulation; spraying the product with or immersing it in a solution; dusting the product with a dry powder of the antimicrobial; applying to the packing material that comes in contact with the food the antimicrobial combined with an organic carrier such as vegetable oil, ethanol, or propylene glycol; or applying multicomponent formulations in a single action (Sofos and Busta, 1992). The method of application to be used for a specific product depends on type of food product; properties of the antimicrobial; type of processing; and, in terms of these factors, convenience of each method. The application method selected should reflect antimicrobial solubility and volatility. Very often, the method used is dictated by what the existing processing and packaging procedures are and how they would allow application of the antimicrobial without disrupting the procedures.

The mechanisms through which antimicrobials affect microbial growth are complex and difficult to elucidate (Russell, 1991). Reasons for this complexity include the potential for more than one cell constituent or activity to be affected, and the complexity and nonhomogeneity of the food system in which compounds are used. The interactions among factors, e.g.,

pH, heat, water activity, and oxygen, involved in microbial proliferation or inhibition add to the complexity and difficulty of elucidating mechanisms of antimicrobial activity. But potential sites or modes of activity include inhibition of cell wall synthesis, activity on cell membranes, inhibition of protein synthesis, activity on nucleic acids, inhibition of enzymes, and interference with cell uptake and transport functions (Sofos, 1989b; Sofos and Busta, 1992).

Microorganisms may exhibit, in addition to sensitivity, resistance to antimicrobials, which may be natural or, as indicated earlier, acquired by selection through continuous exposure to low concentrations of these compounds. The activity of antimicrobials also depends on type and group of microorganism. Because they have fewer barriers than gram-negative bacteria do, gram-positive, nonspore-forming bacteria generally are more sensitive to inhibition by antimicrobials. The outer cell membrane of gram-negative bacteria consists of lipopolysaccharides (LPS), proteins, and lipids, which act as barriers to the entrance of chemical agents (Russell, 1991). Damage to these barriers by chemical or physical agents leads to the sensitivity of gram-negative bacteria to antimicrobials.

Traditional Antimicrobials

Antimicrobials that may be classified as *traditional*, with long or frequent use in processed foods, include sugars, salt, and wood smoke (Sofos and Busta, 1992). Natural sugars such as sucrose, fructose, glucose, syrups, and various corn or other products, which generally are useful in foods as sweeteners, flavorings, and fermentable materials, also can exert antimicrobial activity through direct action in binding moisture. Binding increases osmotic pressure and decreases water activity. Antimicrobials can exert indirect activity by serving as substrates in food fermentations and by producing acids and other antimicrobial agents in foods (Foegeding and Busta, 1991). In addition, sugars may contribute to the preservation of foods through interactions with other components or processing procedures, e.g., heating and drying, involved in manufacture.

Direct microbial inhibition requires sugar concentrations exceeding 40 to 50%; for example, a 50% sucrose concentration decreases water activity to 0.935, which inhibits growth of the pathogen *Clostridium botulinum* (Foegeding and Busta, 1991; Sofos and Busta, 1992). Foods preserved with high sugar concentrations include jams, jellies, preserves, syrups, fruit juice concentrates, sweetened condensed milk,

and candies. Certain yeasts and molds, however, have the ability to tolerate or to grow in the presence of high sugar concentrations or decreased water activity. Small concentrations of sugar act as substrates and support growth of various microorganisms, including spoilage and pathogenic microorganisms and those useful in the production of fermented foods.

Fermentation of naturally occurring or added sugars by useful microorganisms results in the formation of acids, alcohols, and other antimicrobial agents in foods (Foegeding and Busta, 1991; Smith and Palumbo, 1981; Sofos and Busta, 1992). Useful microorganisms are those converting raw materials into edible fermented food products of acceptable quality, shelf-life, and safety. Fermented foods, e.g., cheeses, breads, pickles, and sausages, have desirable textural and flavor characteristics and relatively long shelf-lives, for they may contain acids, alcohols, and other metabolic products of microorganisms, such as bacteriocins, that inhibit or inactivate undesirable spoilage or pathogenic bacteria. Useful microorganisms are involved not only in fermented foods but also in conversion of industrial waste raw materials into chemicals of industrial, pharmaceutical, or nutritional importance.

Common salt (sodium chloride) has been used as a flavoring or a preservative in foods since ancient times. Foods treated with salt include meat, fish, cheese, butter, margarine, and brined vegetables. Curing of meat with impure salt led to the use of nitrates and nitrites as additives for the curing and the preserving of meat products (Sofos and Busta, 1980; Sofos et al., 1979). In recent years, because of the potential link between sodium consumption and development of hypertension in certain individuals, there has been a trend to decrease salt in or to eliminate it from processed foods (Sofos, 1984; Sofos and Raharjo, 1994a, b).

Potential partial replacers of salt in meat and other food products include other chloride salts, e.g., potassium chloride, and phosphates (Sofos, 1986, 1989a). If certain levels of potassium chloride are exceeded, however, bitter flavors may result; phosphates also may contribute undesirable textural and flavor effects. Complete elimination of salt from certain foods may be impossible because of its important contribution to product taste and to technological properties such as water and food particle binding (Sofos, 1984). Nevertheless, many present-day food products are manufactured with amounts of salts lower than those used in the past. This change has been feasible due to the expanded use of mechanical refrigeration and packaging and to other methods contributing to prod-

uct preservation and safety (Sofos, 1993).

The amount of salt necessary to decrease water activity to 0.935, which inhibits growth of *Clostridium botulinum*, is 10% in the water phase of a product; but even lower levels are important because they act synergistically with other antimicrobials such as acids, nitrite, sorbate, and benzoate in controlling microorganisms (Sofos, 1984). *Halophilic*, i.e., salt-loving, bacteria and *osmotolerant*, i.e., sugar-tolerating, or osmophilic yeasts and molds, however, can proliferate in the presence of even higher salt concentrations. Pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* also can tolerate high levels of sodium chloride and are able to survive and even to proliferate in products when salt levels exceed 5% (Sofos, 1993).

Exposure to wood smoke, i.e., smoking of foods, is an ancient practice still used in processing. Wood smoke contributes flavor but also may incorporate antimicrobial components into a product. Components with potential antimicrobial activity isolated from smoke include phenolic compounds, formaldehyde, acetic acid, and creosote. Their antimicrobial activity in today's mildly smoked foods probably is weak. The extent of application of wood smoke to foods not only has been decreased in recent years but also is being replaced by the application of refined liquid smoke flavorings isolated from natural wood smoke condensates (Boyle et al., 1988; Sofos and Maga, 1988; Sofos et al., 1988). These preparations are preferred because they are easy to apply uniformly, the concentrations used can be controlled, pollution from crude tar products can be decreased, and polycyclic aromatic hydrocarbons can be removed.

Organic Acid Antimicrobials

Introduction

One of the primary factors affecting microbial growth in foods is acidity, which is measured in terms of *pH*, or negative logarithm of the hydrogen ion concentration of a solution. Most bacteria prefer pH values near neutrality, i.e., pH 7.0, but the range allowing their survival and growth extends from pH values of less than 2.0 to 9.0 or higher (Doores, 1993). Yeasts and molds are more tolerant of lower pH values than bacteria are. Generally, increasing the acidity, i.e., lowering the pH, of foods is an effective way of limiting microbial growth (Sofos and Busta, 1992). The pH levels can be lowered in foods either through acidulant addition or through natural fermentation, result-

ing in production of acid by desirable microorganisms.

Microorganisms such as lactic acid bacteria produce, among other metabolites, organic acids (Dillon and Cook, 1994). Although lactic acid is the main acid produced, others acids include acetic, formic, and propionic (Figure 3.2). Organic acids such as acetic, citric, erythorbic, fumaric, lactic, malic, and succinic also are produced by food associated fungi such as *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* spp. (Dillon and Cook, 1994). Both fermented foods of lowered pH and acidified products allow limited microbial growth, but its extent and composition depend on the types of microorganisms contaminating the food, type and amount of acid, and composition of food, especially its buffering capacity.

Acids inhibit microbial growth by lowering pH, or through the antimicrobial activity of undissociated molecules or anions (Banwart, 1989). As pH decreases, the antimicrobial activity of *short-chain* organic acids, i.e., organic acids with chains of fewer than eight carbon atoms, increases more than that of long-chain acids. As pH decreases and approaches the pKa of a short-chain organic acid, the undissociated shorter molecule is able to enter the microbial cell, where it dissociates, acidifies the cytoplasm, and interferes with chemical transport across the cell membrane or with enzymatic activity (Banwart, 1989; Sofos, 1989b). For the cytoplasm to maintain a constant pH, protons generated in the cytoplasm must be pumped out, and this act disrupts the proton motive force, resulting in interference with the oxidative phosphorylation and nutrient transport systems (Dillon and Cook, 1994; Lili and Piper, 1994; Sofos, 1989b).

The antimicrobial activity of organic acids is enhanced when they occur in mixtures; in this way, their spectrum of activity also is increased. Shorter-chain organic acids inhibit or inactivate both gram-positive and gram-negative bacteria whereas longer-chain organic acids are effective primarily against gram-positive bacteria, for they cannot penetrate the outer membrane of gram-negative bacteria. Monounsaturated fatty acids generally are less inhibitory to microorganisms than saturated fatty acids are (Kabara,

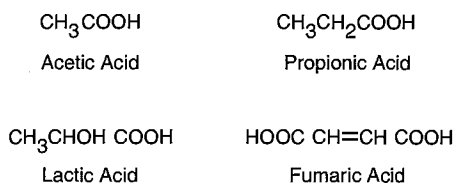


Figure 3.2. Structures of the organic acids, acetic, propionic, lactic, and fumaric, produced by some microorganisms or naturally present in some foods.

1978). Acidic environments not only limit microbial growth but also enhance destruction of microorganisms by heat; thus, decreased pasteurization or sterilization times are possible (Doores, 1993; Foegeding and Busta, 1991). Increased acidity also enhances the antimicrobial activity of the other hurdles, or barriers, involved in food preservation. For example, the antimicrobial activity of preservatives such as nitrites and sorbates increases as product pH decreases (Sofos, 1989b; Sofos and Busta, 1980).

Several naturally occurring organic acids are added to or are formed in foods through microbial processes. Included in this group are acetic, adipic, benzoic, caprylic, citric, fumaric, lactic, malic, propionic, sorbic, succinic, and tartaric acids. Actually, organic acids are commonly added or naturally occurring food preservatives, e.g., benzoic acid in cranberries, propionic acid in Swiss cheese, and sorbic acid in rowanberries. The acids may, in addition to having an antimicrobial effect, act as flavoring agents, buffers, sequesterants, and synergists to antioxidants and curing adjuncts (Foegeding and Busta, 1991). Their antimicrobial activity is higher in acidic environments because their pKa values range from pH 3 to pH 5 (Sofos and Busta, 1992). Their application generally is limited to foods with pH values lower than 6.0 although some, e.g., acetic, lactic, and citric acids, act as acidulants and decrease product pH. Organic acids also have been tested or used as washes, sprays, or dips to decrease the microbial load of meat and poultry carcasses (Sofos, 1994b).

Acetic Acid

Ethanoic or acetic acid (CH_3COOH) is water soluble and has a pKa of 4.75. It is a product of the oxidation of ethanol by *Acetobacter* and *Gluconobacter* bacteria and is prevalent in vinegar. In this form, acetic acid constitutes one of the oldest preservatives in use. A natural process for production of acetic acid may involve an alcoholic fermentation of sugars naturally present in grapes, malts, grains, and other plant materials, followed by natural aerobic oxidation of ethanol to yield acetic acid (Ebner, 1982; Foegeding and Busta, 1991; Sofos and Busta, 1992). Thus, it is found in fermenting plant materials and some dairy products. Acetic acid can be used as a general preservative because it inhibits many species of bacteria and, to a lesser extent, yeasts and molds, and because it is available readily, of low cost, and of low toxicity (Banwart, 1989; Chichester and Tanner, 1972; Sofos and Busta, 1992).

Bacteria inhibited by acetic acid include gram-neg-

ative and gram-positive species, including the spore formers *Bacillus* and *Clostridium*. Pathogenic bacteria inhibited by acetic acid include *Salmonella*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Doores, 1993). Acetic acid derivatives such as sodium and calcium acetate, sodium and calcium diacetate, and dehydroacetic acid also are used as antimicrobial agents. The compounds are GRAS and can be applied to products according to GMPs. In the form of 1.5 to 2.5% solutions, acetic acid can be applied in the decontamination of food animal carcasses (Dickens and Whittmore, 1994; Dickens et al., 1994; Dickson, 1992; Gorman et al., 1995; Hardin et al., 1995; Sofos, 1994b) whereas sodium acetate has exhibited inhibitory activity against *L. monocytogenes* in catfish filets and in sausages (Chang et al., 1995; Rong-Yu et al., 1996; Wederquist et al., 1994, 1995). Acetic acid also has been tested as a vapor at low concentrations (2.7 to 5.4 mg/L) for control of fruit decay by postharvest fungi, and results have been promising (Sholberg and Gaunce, 1995).

Benzoic Acid

The antimicrobial action of benzoic acid ($\text{C}_6\text{H}_5\text{COOH}$) (Figure 3.3), also known as phenylformic or benzenecarboxylic acid, first was described in 1875 (Lück, 1980). The compound is a natural constituent of cranberries, prunes, green-gage plums, apples, strawberries (Figure 3.4), cinnamon, and ripe olives (Chipley, 1993). In addition, benzoic acid may be found in some yogurts as a by-product of microbial growth. Commercially, pure benzoic acid is available as a white granular or crystalline powder with a sweet or somewhat astringent taste and a water solubility of 0.35% at ambient temperature (Sofos, 1994a). Sodium benzoate also is available commercially and has a solubility of more than 50% in water; it thus is more useful in commercial applications than the less soluble acid (Chipley, 1993; Sieber et al., 1995; Sofos and

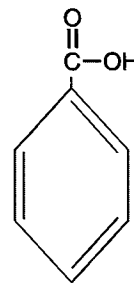


Figure 3.3. Benzoic acid is an antimicrobial acid, which is a natural constituent of cranberries, prunes, green-gage plums, apples, strawberries, and ripe olives.

Busta, 1992).

The antimicrobial activity of benzoate is greater against yeasts than against bacteria and molds but depends on food, pH, water activity, and microorganisms (Sofos, 1994a). Pathogenic bacteria inhibited by benzoate include *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* (Chipley, 1993). Other bacteria inhibited by benzoic acid include *Escherichia coli*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, and *Streptococcus*. Yeasts affected include species in the genera *Candida*, *Debaryomyces*, *Hansenula*, *Rhodotorula*, and *Saccharomyces*. Among the fungi affected are *Alternaria*, *Aspergillus*, *Byssoschlamys*, *Mucor*, *Penicillium*, *Rhizopus*, and *Neosartorya fischeri*. Spraying beef carcasses with a 0.7% benzoic acid solution eliminated some species of fungi, but others were unaffected or grew better on the treated carcasses (Nassar et al., 1995). Concentrations effective in inhibiting microorganisms are in the range of 0.05 to 0.1% for yeasts and molds and 0.01 to 0.2% for bacteria.

As with other organic acids, the antimicrobial activity of benzoic acid is due mostly to the undissociated molecule, even though the dissociated compound also has shown antimicrobial activity (Eklund, 1988, 1989). In general, antimicrobial activity increases as the pH value of the food decreases near to the pKa of 4.19, and maximal antimicrobial activity occurs at pH values of 2.5 to 4.0; at pH 6.0, antimicrobial activity is only 1% of that at pH 4.0 (Sofos, 1994a).

Benzoate can be metabolized by bacteria such as the *Enterobacteriaceae*, *Pseudomonas*, and *Corynebacterium glutamicum* and by a thermophilic *Bacillus* employing a pathway involving β -ketoapitate (Chipley, 1993; Sofos, 1994a). Certain species of yeasts are resistant to inhibition by benzoic acid; these include *Byssoschlamys nivea*, *Pichia membranaefaciens*, and *Talaromyces flavus*. Osmotolerant species of yeasts, such as *Zygosaccharomyces bailii*, which spoil

intermediate moisture foods, also are resistant to benzoate (Wind and Restaino, 1995). Other microorganisms that can acquire resistance to benzoic acid include *Nectria galligena*, *Gluconobacter oxydans*, and *Escherichia coli* (Chipley, 1993). Variation of yeast species in resistance to benzoic acid could be due to differences in benzoic acid uptake rate by the microbial cell, to the capacity of the cell to remove the molecule, or to its sensitivity to benzoic acid (Warth, 1989a). The most important of these mechanisms, however, may be the ability of yeasts to remove benzoic acid from the cell. This acquired resistance is attributed to an inducible, energy-requiring system that transports the benzoate molecule out of the microbial cell (Warth, 1989b, c). Benzoic acid is a GRAS substance and is tolerated well by humans (Chichester and Tanner, 1972; Chipley, 1993; Sofos, 1994a; Sofos and Busta, 1992).

Lactic Acid

Lactic, or 2-hydroxypropanoic ($\text{CH}_3\text{CHOHCOOH}$), acid (pKa = 3.83) is formed by bacteria in lactic acid fermentations. Common bacteria producing lactic acid from sugar include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, and *Leuconostoc*, as well as certain molds. Heterofermentative organisms, e.g., *Leuconostoc*, also produce other end-products such as acetic acid, ethanol, carbon dioxide and diacetyl, as well as bacteriocins. Homo- and heterofermentative fermentations result in products such as cheese, sauerkraut, pickles, olives, and fermented meat. The lactic acid formed in these products through microbial degradation of sugars decreases the pH to levels unfavorable for growth of spoilage or pathogenic bacteria (Sofos and Busta, 1992).

Microorganisms inhibited by lactic acid and lactates include *Clostridium botulinum*, *C. perfringens*, *C. sporogenes*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, *Serratia liquefaciens*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, and *Enterobacter cloacae* (Doores, 1993; Harmayani et al., 1993; Houtsma et al., 1994; Maas et al., 1989; Meng and Genigeorgis, 1994; Miller and Acuff, 1994; Pelroy et al., 1994; Shelef and Yang, 1991). Inhibition of microorganisms such as *Brochothrix thermosphacta*, *Salmonella typhimurium*, and *Pseudomonas fragi* by sodium lactate depends on substrate properties and environmental conditions (Grau, 1980a, b; Harmayani et al., 1991; Maas, 1993). Certain bacteria such as *Escherichia coli* O157:H7 possess an inducible acid tolerance response. Lactic acid (3%) used in combination with potassium sorbate (5%)



Figure 3.4. Strawberries are one of the fruits containing benzoic acid.

as a solution extended the shelf-life of fresh, vacuum packaged poultry meat stored under refrigeration (Kolsarici and Candogan, 1995). Overall, the antimicrobial activity of lactic acid ranges from good to poor, depending on the substrate (Banwart, 1989). The sodium and potassium salts, i.e., lactates, of lactic acid are produced chemically after a natural microbial fermentation for production of lactic acid and have been used relatively extensively in recent years as sensory potentiators, flavorings, and antimicrobial agents in foods such as processed meats and poultry products (Shelef, 1994; Wederquist et al., 1994, 1995).

Lactic acid, which is highly soluble in water and is a GRAS substance, usually is produced commercially by microbial fermentation. In addition to being an acidulant and antimicrobial, lactic acid as well as its salts, i.e., sodium, calcium, and potassium, is used as a flavoring agent or emulsifier in food products (Sofos and Busta, 1992) as well as an agent in solutions to decontaminate food animal carcasses or portions (Hardin et al., 1995; Sofos, 1994b). Commercially, lactic acid is available as a hygroscopic, syrupy liquid of moderately strong acid taste and is used in beverages, jams, jellies, sherbets, and confectionery products.

Propionic Acid

Propionic ($\text{CH}_3\text{CH}_2\text{COOH}$) acid ($\text{pK}_a = 4.88$), an aliphatic, monocarboxylic acid, is an oily liquid with a strong, pungent, rancid odor. Propionic acid characteristically is produced by bacteria belonging to the genus *Propionibacterium*, as in the ripening of Swiss-type cheeses. This natural acid acts as a flavoring agent and mold inhibitor in Swiss cheeses. In addition, propionic acid is formed by bacteria in the gastrointestinal tracts of ruminant animals. In the pure form, it is miscible in water, alcohol, ether, and chloroform and is somewhat corrosive (Doores, 1993; Sofos, 1994a; Sofos and Busta, 1992). The sodium and calcium salts, available commercially as white, free-flowing powders, are used as food preservatives.

Propionates are active inhibitors of molds and rope-forming bacteria (*Bacillus subtilis*) in bread (Figure 3.5), but their activity against yeasts is limited; some yeasts can catabolize the compounds. Propionate-resistant yeasts can be detected from several foods, including fish (Walters and Levin, 1994). Propionates are more effective inhibitors of mold growth than benzoates are. Microorganisms inhibited by propionates include spore forming bacteria such as *B. subtilis* (*mesentericus*) as well as *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Proteus vulgaris*, *Lactobacillus plantarum*, *Pseudomonas*

spp., *Sarcina lutea*, *Aspergillus* spp., *Torula* spp., *Trichophyton mentagrophytes*, and *Saccharomyces ellipsoideus* (Doores, 1993).

The extent of antimicrobial activity is affected by the type of microbial species and the pH of the food to be preserved. The antimicrobial activity of propionic acid in minced pork generally was greater than that of lactic acid (Ogden et al., 1995). As with other lipophilic acid preservatives, antimicrobial activity of propionate is higher as pH decreases towards its pK_a (Foegeding and Busta, 1991). Inhibition of *Escherichia coli* may be reversed with addition of β -alanine, a fact perhaps indicating that propionate interferes with synthesis of β -alanine (Doores, 1993). Addition of β -alanine, however, did not reverse the inhibitory effect of propionic acid against *Aspergillus clavatus*, *Bacillus subtilis*, *Pseudomonas* spp., or *Trichophyton mentagrophytes*. Propionate also may exert its activity through interference with nutrient transport and inhibition of enzyme systems, as other lipophilic acid antimicrobials do (Sofos, 1989b, 1994a; Sofos and Busta, 1992).

Propionic acid and its sodium and calcium salts are GRAS for use in food products. Because it is metabolized as a fatty acid, propionate in concentrations as high as 1%, which may be present in Swiss-type cheeses, has no adverse effects on the health of the general population. The FAO of the United Nations has set no limits on the ADI of propionate for humans. Levels used in food preservation are in the range of 0.1 to 0.38%.



Figure 3.5. Propionates are added to bread where they are active inhibitors of molds and rope-forming bacteria.

Sorbic Acid

Sorbic acid (Figure 3.6) is another naturally occurring antimicrobial substance available commercially as a synthetic compound. In the 1800s, sorbic acid was isolated first from the oil of unripened rowanberries; the first patent for its use as an antimicrobial agent was issued in 1945 (Sofos, 1989b; Sofos and Busta, 1981). Chemically, sorbic—or 2,4-hexadienoic—acid is a trans-trans unsaturated fatty acid with two highly reactive conjugated double bonds and a carboxyl group. The carboxyl group of sorbic acid yields salts and esters, which when dry are stable to oxidation but when in solution are relatively unstable (Sofos and Busta, 1992, 1993). Common derivatives of sorbic acid are the potassium, calcium, and sodium salts; other derivatives include alcohols, aldehydes, esters, and amide derivatives such as sorbohydroxamic acid, sorboyl palmitate, sorbamide, methyl sorbate, ethyl sorbate, and sorbic anhydride (Sofos, 1994a).

Sorbic acid is available commercially as a white, free-flowing powder or as granules with a weak acrid odor and acid taste. The potassium, sodium, and calcium salts are available as fluffy, odorless, and tasteless powders or granules (Lück, 1980). The solubility of the acid in water is only 0.15%; its potassium, sodium, and calcium salts have solubilities of 58.2, 32, and 1.2%, respectively.

Sorbates are effective antimicrobial agents against a variety of yeasts, molds, and bacteria at concentrations of 0.05 to 0.3% (Sofos, 1989b). Among the species of yeasts inhibited by sorbate are those belonging to the genera *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Endomycopsis*, *Hansenula*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Torulasporea*, *Torulopsis*, and *Zygosaccharomyces* (Sofos and Busta, 1993). Sorbates inhibit yeasts in fermented vegetables, fruit juices, wines, dried fruits, cheeses, fish, and meats. Specific products in which sorbates are applied for inhibition of yeasts are carbonated beverages, salad dressings, syrups, tomato products, jams, candies, jellies, and chocolate syrups (Liewen and Marth, 1985; Sofos and Busta, 1993).

Sorbates also are effective inhibitors of many molds, including species of *Alternaria*, *Ascochyta*, *Aspergillus*, *Botrytis*, *Cephalosporium*, *Cladosporium*,

Colletotrichum, *Cunninghamella*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Helminthosporium*, *Heterosporium*, *Humicola*, *Monilia*, *Mucor*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Pullularia*, *Rhizoctonia*, *Rhizopus*, *Rosellinia*, *Sporotrichum*, *Trichoderma*, and *Truncatella* (Sofos, 1989b; Sofos and Busta, 1993). Mold inhibition by sorbates is important in cheeses as well as in butter, fruits and fruit juices, grains, breads, cakes, smoked fish, and dry sausages. In addition to inhibiting mold growth, sorbates inhibit mycotoxin formation, except when subinhibitory levels of sorbates actually may stimulate mycotoxin production (Liewen and Marth, 1985). Conditions allowing mycotoxin formation depend on mold strain, sorbate level, and storage temperature, among other factors (Sofos, 1989b).

The antimicrobial activities of sorbates against bacteria differ, but many species are inhibited, including gram-positive and gram-negative, catalase-positive and catalase-negative, aerobic and anaerobic, mesophilic and psychrotrophic species (Sofos, 1994a; Sofos and Busta, 1993; Sofos et al., 1986). Species of bacteria inhibited by sorbate belong to the genera *Acetobacter*, *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Campylobacter*, *Clostridium*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Listeria*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Pediococcus*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Staphylococcus*, *Vibrio*, and *Yersinia* (Sofos and Busta, 1993).

Certain yeasts, molds, and bacteria are resistant to inhibition by sorbate or are able to metabolize it through biochemical pathways similar to those used for other fatty acids (Sofos, 1989b; Sofos and Busta, 1993). Strains of molds belonging to genera such as *Penicillium*, *Aspergillus*, and *Mucor* have been shown to decompose sorbate in cheese and fruit products, depending on microbial load, prior exposure to subinhibitory levels of sorbate, sorbate concentration, and food product type. A common quality defect resulting from the metabolism of sorbate by molds in cheeses is the development of a kerosene or plastic paint-type odor due to accumulation of the chemical 1,3-pentadiene (Liewen and Marth, 1985; Sofos, 1989b). Lactic acid bacteria also have degraded sorbate in wine and fermented vegetables, resulting in production of various by-products (ethyl sorbate, 4-hexanoic acid, 1-ethoxyhexa-2,4-diene, and 2-ethoxyhexa-3,5-diene) (Sofos and Busta, 1993), some of which impart undesirable sensory properties.

The antimicrobial activity of sorbate, as of other lipophilic acid preservatives, increases as the pH value of the food decreases toward the pKa of 4.76. Un-

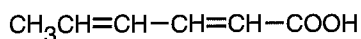


Figure 3.6. Sorbic acid is an effective antimicrobial agent against a variety of yeasts, molds, and bacteria.

der experimental conditions, measurable microbial inhibition is detected, even at pH values in the range of 6.5 to 7.0. These pH maxima for microbial inhibition by sorbate are higher than those for benzoate or propionate. Potassium sorbate is a more effective growth inhibitor against *Zygosaccharomyces bailii* in salsa mayonnaise stored at 23 to 25°C than sodium benzoate is (Wind and Restaino, 1995). Although the undissociated form of the acid is known as the highly active species, microbial inhibition also has been attributed to the dissociated molecule; but its activity is 10 to 600 times less than that of the undissociated acid (Eklund, 1983; Sofos, 1994a).

Commonly used concentrations (0.02 to 0.3%) of sorbate in food preservation are adequate only for inhibition of microbial proliferation and have no lethal antimicrobial effects. The molecular mechanisms of microbial inhibition are not well defined, but sorbates are known as inhibitors of bacterial spore germination through inhibition of spore-lytic enzymes or through interactions with spore membranes (Sofos et al., 1986). Inhibition of microbial cell metabolism by sorbate may be attributed to several mechanisms including alterations of cell morphology; alterations of morphology, integrity, and function of cell membranes; inhibition of cell transport and nutrient uptake; and inhibition of enzymatic activity (Sofos, 1989b, 1994a; Sofos and Busta, 1993; Sofos et al., 1986). Effects observed have included inhibition of the electron transport system and inhibition of the proton motive force existing across microbial cell membranes, which is necessary for nutrient uptake by the cell (Eklund, 1985; Sofos, 1989b, 1994a).

Sorbic acid and its potassium, calcium, and sodium salts are GRAS when GMPs are used. Overall, sorbates have fewer negative toxicological effects than many other commonly used food additives do. For instance, the dose of sorbate proving lethal to experimental animals is lower than that of common table salt (Sofos, 1989a, 1994a).

Other Organic Acids

Several other organic acids are present as natural constituents in different foods and have found some uses as intentional additives in food processing and preservation. These acids include adipic, ascorbic and isoascorbic, caprylic, citric, formic, fumaric, malic, succinic, and tartaric acids (Figure 3.7). Natural sources for one or more of these compounds include apples, apricots, cherries, bananas, carrots, broccoli, grapes, peas, potatoes, peaches, rhubarb, citrus fruits, figs, tomatoes, beans, asparagus, beets, sauerkraut,

cheese, and other fermented foods (Figure 3.8) (Doores, 1993; Gardner, 1972; Foegeding and Busta, 1991; Sofos and Busta, 1992).

Adipic acid ($\text{COOH}(\text{CH}_2)_4\text{COOH}$), a nonhygroscopic compound of low water solubility, is useful commercially as an acidulant and buffer or neutralizing agent in dry, powdered food products. Inhibition of microorganisms is due only to its acidifying activity. In the United States, it is GRAS and because of its low hygroscopicity is used in baking powders, powdered fruit beverages, candies, biscuits, gelatin desserts, oils, and process cheeses (Gardner, 1972; Sofos and Busta, 1992).

Ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), or vitamin C, is present

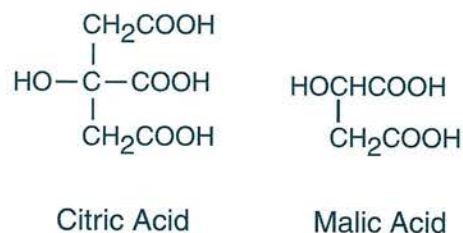


Figure 3.7. Citric and malic acids are two of many naturally occurring acids in many fruits and vegetables that are antimicrobial.

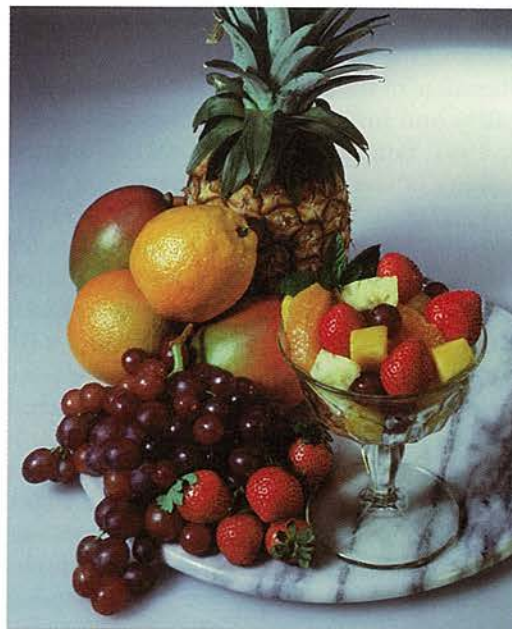


Figure 3.8. Many fruits contain naturally occurring acids that have an antimicrobial effect.

naturally in many foods and together with its isomer, isoascorbic or erythorbic acid, and their salts is highly soluble in water and safe for use in foods. When used alone, however, these compounds are only weakly inhibitory against microorganisms, with the exception of *Pseudomonas* spp. in liquid products and, when used in combination with citric acid, *Clostridium botulinum* is inhibited in cooked potatoes (Banwart, 1989; Notermans et al., 1985). When used in cured meats to accelerate the curing reaction, levels of approximately 0.05% enhance depletion of nitrite and therefore decrease formation of carcinogenic nitrosamines, whereas levels of approximately 0.02% enhance the inhibitory activity of nitrite against *C. botulinum* (Sofos and Raharjo, 1994a; Tompkin, 1993).

Caprylic, or octanoic, acid ($\text{CH}_3(\text{CH}_2)_6\text{COOH}$) is a colorless, oily liquid with a slightly unpleasant odor and a burning, rancid taste and is only slightly soluble in water (Doores, 1993). Its antimicrobial activity is limited, depending on pH of the food (Kabara et al., 1972a, b; Woolford, 1975). The minimal concentration inhibiting *Vibrio parahaemolyticus* in culture broth was 0.01% (Beuchat, 1980). Caprylic acid is approved as a GRAS substance and may be used as a flavoring adjuvant or mold inhibitor at levels of 0.001 to 0.16% in baked goods, cheeses, fats, oils, frozen dairy desserts, puddings, soft candies, snack foods, meat products, and cheese wrappers (Doores, 1993; Sofos and Busta, 1992).

Citric acid ($\text{COOHCH}_2\text{C}(\text{OH})(\text{COOH})\text{CH}_2\text{COOH}$) is the major acid present in citrus fruits. It is used widely as an acidulant in carbonated beverages and other foods because of its unique and pleasant sour flavoring ability and high water solubility (Foegeding and Busta, 1991; Sofos and Busta, 1992). The antimicrobial activity of the compound generally is due to low pH. The compound inhibits bacteria causing *flat-sour spoilage*, i.e., spoilage whereby no gas is formed, particularly in tomato juice. It also inhibits *Salmonella typhimurium* in skim milk and poultry carcasses; and *Pseudomonas fluorescens* on beef carcasses (Doores, 1993). Citrate also is used as a food emulsifier. Foods in which citric acid is approved for use include carbonated beverages, fruit preserves, jams, jellies, canned vegetables, dairy products, salad dressings, ice creams, sherbets, and ices. Citric acid is a less effective antimicrobial than other acids are because many microorganisms are able to metabolize it and because of its low ($\text{pK}_1 = 3.14$ and $\text{pK}_2 = 4.77$) pKa value (Foegeding and Busta, 1991).

Fumaric acid ($\text{COOHCH}=\text{CHCOOH}$) is a nonhygroscopic, monounsaturated dicarboxylic compound of low water solubility, and is found naturally in many

plants (Deshpande et al., 1994). It has a strong acidic taste but blends with certain flavoring compounds to intensify aftertaste (Doores, 1993; Sofos and Busta, 1992). When added to a food, fumaric acid imparts acidity as well as antioxidant activity (Gardner, 1972). Its antifungal properties are greater than those of acetic, citric, malic, or tartaric acids. A concentration of 0.5% inactivated ascospores of *Talaromyces flavus* in 20 minutes (min) at 80°C. This lethal effect increased as the pH decreased from 5.0 to 2.5 whereas concentrations of 2.5% malic, citric, and tartaric acids were not lethal (Beuchat, 1988). Similar lethal activity was found against *Neosartorya fischeri* (Conner and Beuchat, 1987). Mono- and di-methyl and ethyl esters of fumaric acid (0.125 to 0.2%) inhibited growth of *Clostridium botulinum* in canned bacon, but the acid itself was only slightly inhibitory (Huhtanen, 1983). Also, n-monoalkyl fumarates and maleates esterified with C_{13} to C_{18} alcohols showed antimicrobial activity whereas methyl-n-alkyl fumarates and di-n-alkyl fumarates were slightly active or inactive (Dymicky et al., 1987). In wines, fumaric acid (0.15%) is used as an acidulant and inhibitor of the malolactic fermentation (Doores, 1993). Commercially, fumaric acid is used in fruit drinks, pie fillings, gelatin desserts, doughs, biscuits, and wines (Doores, 1993; Sofos and Busta, 1992).

Malic acid ($\text{COOHCH}_2\text{CHOHCOOH}$), also known as hydroxysuccinic or 1-hydroxy-1,2-ethanedicarboxylic acid, is the predominant acid in many fruits, vegetables, and legumes, including apples, cherries, apricots, grapes, peaches, oranges, bananas, broccoli, carrots, peas, potatoes, rhubarb, citrus fruits, figs, beans, and tomatoes (Foegeding and Busta, 1991). Malic acid is nonhygroscopic, has high water solubility and a strong acidic flavor, but does not impart as much sour taste as other acids do (Doores, 1993). Its antimicrobial activity generally is due to its acidifying ability, but it is used mostly for its esterification and flavoring properties. As a GRAS substance, it is used in products such as fruit preserves, jams, jellies, sherbets, ices, beverages, and salad dressings (Doores, 1993; Sofos and Busta, 1992).

Succinic acid ($\text{COOH}(\text{CH}_2)_2\text{COOH}$), a normal constituent of almost all plant and animal tissues, is nonhygroscopic and of low water solubility, with a slightly bitter taste; it may, however, be used as a flavor enhancer and acidulant in gelatin desserts, baked goods, and cake flavorings (Deshpande et al., 1994; Gardner, 1972; Sofos and Busta, 1992). One study found that it decreased total bacteria and *Salmonella* counts in fresh poultry meat but adversely affected product appearance (Cox et al., 1974).

Tartaric acid ($\text{COOH}[\text{CHOH}]_2\text{COOH}$) is common in grapes. It is highly soluble in water and has a strong, tart taste that enhances grape-like flavors. It acts synergistically with antioxidants to minimize lipid oxidation, product rancidity, and discoloration in cheeses, and also inhibits microbial growth by means of acidification. As a GRAS compound, it is used in fruit products such as jams, jellies, preserves, sherbets, and grape flavored beverages and in baked products in the form of cream-of-tartar (monopotassium tartrate) (Doores, 1993; Sofos and Busta, 1992).

Lipid Antimicrobials

Introduction

Fatty acids and their soaps have been used since antiquity for cleansing and disinfecting. Furthermore, fatty acids and their polyhydric alcohol esters are considered to be of low toxicity and have been used intentionally, mostly as emulsifiers, in foods since the early 1900s (Kabara, 1993). At low concentrations, the compounds inhibit certain bacteria and fungi in some foods and may have considerable potential for commercial use as safe preservatives.

In general, fats and oils from animals and vegetables are known for their ability to inhibit microorganisms through antimicrobial agents such as fatty acids or their oxidation products, such as peroxides, and through associated antioxidant phenolic compounds formed by plants (Dallyn, 1994). The antimicrobial activity of lipid oxidation products has been known since the 1930s, when increased inhibition of microorganisms was observed in foods with oils containing more unsaturated and longer-chain fatty acids after exposure to ultraviolet light (Harris et al., 1932). Oxidation of lipid substances is unacceptable in food products, however, because it results in undesirable rancid odors, off-flavors, and oxidized colors. Another property of fats and oils of microbiological importance is their ability to protect microorganisms from thermal destruction. As a result, foods require extended heating for uniform heat penetration and distribution (Ababouch and Busta, 1987; Ababouch et al., 1987).

Fatty Acids

Fatty acids, as components of natural fats, are primarily even numbered straight-chain molecules, often with one or more double (ethylenic) or triple (acetylenic) bonds. The fatty acid compositions of natural

fats differ considerably, depending on origin (Beuchat and Golden, 1989). Plant oils, e.g., coconut oil, often contain an abundance of medium-chain fatty acids, and oils from marine animals and plants often contain an abundance of unsaturated fatty acids. Animal fats are more saturated.

As indicated earlier, *short-chain fatty acids*, i.e., fatty acids with 6 carbon atoms, such as acetic and lactic acids, show inhibitory activity at relatively high concentrations (1 to 3%) against gram-positive and gram-negative bacteria, as well as against fungi (Doores, 1993; Eklund, 1989; Sheu et al., 1975). These short-chain fatty acids are common by-products of microbial fermentation but are relatively uncommon in the natural fats of plants and animals, with the exception of the fats of ruminant animals. Medium-chain saturated fatty acids (C_8 to C_{14}) and their potassium and sodium salts are inhibitory mainly towards gram-positive bacteria and yeasts. Inhibition generally occurs in media with concentrations from 0.0005 to 0.005%, but higher concentrations usually are required in foods. Lauric acid usually is the most inhibitory fatty acid against gram-positive organisms whereas capric acid is most active against yeasts (Kabara, 1978, 1993; Nieman, 1954).

The antimicrobial effect of saturated fatty acids generally is caused by the undissociated form of the molecule, and activity in foods therefore is controlled by pH, for this determines the extent of dissociation. Activity usually is highest in acidic ($\text{pH} \leq 4.6$) and mildly acidic ($\text{pH} \sim 5.5$) foods. Although gram-negative bacteria generally are less susceptible to medium-chain fatty acids, certain families, including some *Enterobacteriaceae* and *Neisseriaceae*, can be inhibited, especially if sensitized by chemical or physical treatments (Galbraith and Miller, 1973; Galbraith et al., 1971; Sheu and Freese, 1973; Tsuchido and Takano, 1988).

Sheu and Freese (1972, 1973) showed that the gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* were inhibited by short-chain but not by medium- or long-chain fatty acids at concentrations strongly inhibiting gram-positive *Bacillus subtilis*. Cell surface mutant cultures of *E. coli* and *S. typhimurium* with altered membrane lipopolysaccharides (LPS) became sensitive to inhibition, indicating that the LPS layer acts as a protective barrier against penetration of fatty acids into the cytoplasmic membrane.

Branched-chain and hydroxylated fatty acids possess slightly less antimicrobial activity than their straight-chain counterparts do (Kabara, 1993). The influence of chain length and structure on effective-

ness probably is balanced by properties such as solubility, hydrophobicity, and configuration, which affect ability to react with the microbial cell membrane. Fatty acids with chain lengths greater than C_{14} are not sufficiently soluble in the suspending solution for adequate cell contact and prolonged exposure. Similarly, introduction of hydrophobic groups, such as phenyl rings, decreases inhibitory activity due to their insolubility whereas increasing polarity with a hydroxyl or an amine group restores this activity (Bayliss, 1936; Kabara, 1993).

The presence of unsaturated linkages can markedly increase the antimicrobial activity of fatty acids with 1, 2, or 3 double bonds, e.g., oleate, linoleate, and linolenate, over that of saturated stearate (Kodicek, 1949; Nieman, 1954). The magnitude of inhibition is influenced by degree and position of unsaturation. Kabara (1993) concluded that the most active monounsaturated fatty acid is palmitoleic whereas linoleic is the most active polyunsaturated fatty acid (Kabara, 1993; Kabara et al., 1972a, b). The *cis* forms of fatty acids are active whereas the *trans* isomers are inactive (Bayliss, 1936; Kodicek, 1949), probably because steric hindrance of straight-chain acids prevents contact with cell membranes. Exogenously added *cis*-unsaturated acids act on lipid acyl chains in lipid bilayers and disrupt the membranes, but *trans*-unsaturated and saturated fatty acids have little effect (Anel et al., 1993).

These facts support the explanation that the plasma membrane is the likely site of action. In addition to direct interaction of the undissociated molecule with the cell membrane, unsaturated fatty acids may exert some antimicrobial activity by autoxidation, which results in the formation of peroxides and other active oxygen metabolites. This oxygen dependent activity would be expected to be relatively pH independent under physiological conditions and to be most active against anaerobes and other organisms lacking enzymatic defenses against active oxygen metabolites.

Unsaturated fatty acids are well known to exert an antibacterial effect on gram-positive bacteria, acid-fast bacteria, and yeasts (Nieman, 1954); but most gram-negative bacteria are resistant. Unsaturated fatty acids, particularly oleic, linoleic, and linolenic acids, are inhibitory at 0.005 to 0.02% in microbiological media against gram-positive cocci, lactobacilli, corynebacteria, and endospore forming bacilli, i.e., *Bacillus* and *Clostridium* (Nieman, 1954). Foster and Wynne (1948a, b) observed that spore germination by *C. botulinum* was inhibited by as little as 0.01 to 0.1% of oleic, linoleic, or linolenic acid. Ababouch et al.

(1992) reported that linolenic acid was the most inhibitory unsaturated fatty acid and that lauric acid was the most inhibitory saturated acid against *C. botulinum*, *C. sporogenes*, and *B. cereus*.

Fatty acids also have antimicrobial activity against certain fungi (Nieman, 1954; Wyss et al., 1945). As with bacteria, antifungal activity depends on chain length, cell and fatty acid concentration, and pH and composition of the food. Saturated fatty acids of chain lengths C_4 to C_{10} were most active against the molds *Aspergillus niger*, *Trichoderma viride*, *Myrothecium verrucaria*, and *Trichophyton mentagrophytes*; other studies have shown that fatty acid activity against fungi increased as chain length increased up to 10 carbons, a length one or two carbons shorter than optimum for bacteria (Kabara, 1993).

The antimicrobial activity of fatty acids can be negated by certain substances in media and foods. Proteins such as serum albumin bind and neutralize fatty acids (Dubos and Davis, 1946). Starch also neutralizes the inhibitory effect of fatty acids on spore formation and germination (Foster and Wynne, 1948a, b; Hardwick et al., 1951). Substances such as cholesterol, lecithin, saponin, calcium ions, magnesium ions, and charcoal also antagonize fatty acid inhibitory activity. In foods, lipophilic compounds can be sequestered in the fatty phases of foods and become unavailable for contact with microorganisms. Certain microorganisms, especially fungi and some bacterial groups, can metabolize fatty acids and thereby eliminate antimicrobial activity. The practical significance of antimicrobial activity from fatty acids in many foods therefore is unknown or questionable.

Monoacylglycerols and Other Fatty Acid Esters

Esterification of fatty acids with polyhydric alcohols or with sugars yields compounds with considerable antimicrobial activity and the ability to function as emulsifiers in foods (Conley and Kabara, 1973; Kabara et al., 1972b; Shibasaki, 1982). In general, monoacylglycerols, i.e., monoglycerides, formed through the reaction of medium-chain fatty acids with glycerol, have more potent antimicrobial properties and show a wider spectrum of activity than free fatty acids do. Antimicrobial activity has been demonstrated for esters formed between fatty acids and a variety of polyhydric alcohols or compounds possessing hydroxyl groups, e.g., sugars and peptides. The requirement for antimicrobial activity seems to be that a hydrophilic group be attached to the lipid component, because esterification with monohydric alcohols yields inactive

compounds (Conley and Kabara, 1973).

In most instances, the monoacylglycerol is a more potent antimicrobial than the corresponding free fatty acid (Kabara, 1993). Lauric acid and palmitoleic acid form the saturated and unsaturated monoacylglycerols most inhibitory against bacteria (Conley and Kabara, 1973), but their effectiveness depends on the food system (Kabara, 1993; Wang and Johnson, 1992, 1997; Wang et al., 1993). Although monoacylglycerols containing unsaturated fatty acids could be very active antimicrobial agents, synthesizing and stabilizing this group of compounds is difficult. In addition to the problem of autoxidation, isomerization can occur from *cis* to *trans*, thereby canceling antimicrobial activity. Shibasaki (1982) concluded that lauric acid and its glycerol, sucrose, and polyoxyethylene esters seemed to have greater antimicrobial activity than other hydroxyl compounds tested did. The monoacylglycerols containing C₁₀ and C₁₂ fatty acids and sugar esters of C₈ to C_{18:1} fatty acids were most active against gram-positive bacteria and certain yeasts (Shibasaki, 1982).

Monoacylglycerols are especially active against gram-positive bacteria and certain fungi and have little activity against gram-negative bacteria. Several studies have indicated that many gram-positive bacteria, including *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *S. epidermidis*, streptococci groups A and D, *Nocardia asteroides*, *Micrococcus* spp., *Pneumococcus* spp., and *Corynebacterium* spp. are sensitive to low concentrations of monolaurylglycerol (monolaurin) in microbiological culture media (Conley and Kabara, 1973; Kabara et al., 1972a, b; Kabara et al., 1977; Kato and Shibasaki, 1975) (Figure 3.9). Monolaurylglycerol prevents or delays growth and toxin formation by *S. aureus* and *Streptococcus* spp. (Schlievert et al., 1992). Antimycotic activity of monolaurylglycerol was demonstrated against *Aspergillus niger*, *Penicillium citrinum*, *Saccharomyces cerevisiae*, *Candida utilis*, *C. albicans*, *Cladosporium* spp., and *Alternaria* spp. (Kabara et al., 1977; Kato and Shibasaki, 1975; Marshall and Bullerman, 1986).

Fatty acids and monoacylglycerols were evaluated

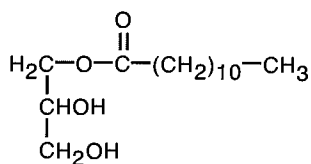


Figure 3.9. Monolaurin, a fatty acid ester, that may provide an antimicrobial effect in foods.

for antimicrobial activity against a strain (Scott A) of *Listeria monocytogenes* in media (Wang and Johnson, 1992; Wang et al., 1993) and in foods (Wang and Johnson, 1997). The C_{12:0} and C_{18:3} acids and monolaurylglycerol were bactericidal in culture broth at 0.001 to 0.002%, compared with potassium salts of conjugated linoleic acids (CLA) and C_{18:2}, which were listericidal at 0.005 to 0.02%. Acids of C_{14:0}, C_{16:0}, C_{18:0}, C_{18:1}, and monomyristylglycerol and monopalmitylglycerol were not inhibitory at 0.02%. The bactericidal activity in brain heart infusion broth was higher at pH 5 than at pH 6. Monoacylglycerols were synthesized from coconut oil and milkfat by lipase catalyzed glycerolysis and evaluated for inhibition of *L. monocytogenes* (Wang and Johnson, 1992). The monoacylglycerol fraction from coconut, but not from milkfat, had strong inhibitory activity associated with fatty acids of the series C₈ to C₁₄, with the C₁₂ and C₁₀ seeming most active. Monoacylglycerols also were demonstrated to inhibit *L. monocytogenes* in foods, particularly foods with reduced fat content (Wang and Johnson, 1997).

Monacylglycerols also have been reported to inhibit certain gram-negative organisms, but the spectrum of activity clearly is more limited than that for gram-positive bacteria. Beuchat (1980) found that monolaurylglycerol was more active than the shorter- (C₈ to C₁₀) or longer- (C₁₄) chain monoacylglycerols toward *Vibrio parahaemolyticus*, a gram-negative marine bacterium. The minimal inhibitory concentration for monolaurylglycerol (0.0005%) was lower than that for sodium benzoate (0.03%) or sorbic acid (0.007%). Kato and Shibasaki (1975) also reported on the inhibitory activity of monoacylglycerols toward gram-negative bacteria. Activity was enhanced by chelating agents, which probably disrupted the LPS layer of the cell membrane and thus allowed penetration of the outer membrane by monoacylglycerides. Takano et al. (1979) found that freezing *Salmonella typhimurium* or other gram-negative bacteria decreased viability in the presence of various lipophilic compounds including sodium laurate and monolaurylglycerol. Monoacylglycerols thus can be antimicrobial to gram-negative bacteria, especially if the cell surface is altered by chemical or physical treatments.

Esters formed from natural alcohols more complex than glycerol also are antimicrobial. Sucrose polyesters have been approved as emulsifiers for application in various foods. Early studies indicated that sucrose esters of lauric acid were inhibitory mainly to gram-positive bacteria and fungi but also had some inhibitory effect on gram-negative bacteria (Conley and Kabara, 1973; Kato and Arima, 1971). Of the sucrose

fatty acid esters tested, sucrose dicaprylate was most active but still was less active than monolaurylglycerol (Beuchat, 1980; Kato and Shibasaki, 1975). Sucrose esters are heat stable and, because they can withstand autoclaving, are useful in stabilizing canned or other heat treated foods especially because they seem active against sporeformers including *Bacillus stearothermophilus*, *B. coagulans*, *Desulfotomaculum nigrificans*, and several *Clostridium* spp.

Marshall and Bullerman (1986) did not detect antimicrobial activity of six sucrose esters formed with a mixture of the palmitic and stearic acids against growth of *Saccharomyces cerevisiae* or against growth and acid production by several lactic acid bacteria, but they did detect antimycotic activity towards several mold species in the genera *Aspergillus*, *Penicillium*, *Cladosporium*, and *Alternaria*. Esters of fatty acids also have been synthesized from linear polymers of glycerol (Babayan et al., 1964). Generally, as the hydrophilic group became larger, the molecules became more selective in their activity. It seemed that, regardless of the hydrophilic moiety of the ester, overall antimicrobial activity was determined according to fatty acid component, with medium-chain fatty acids being most active (Conley and Kabara, 1973).

Lipopeptides

Lipopeptides, which are derived from natural components by means of the condensation of peptides or amino acids and fatty acids, would be expected to have excellent antimicrobial properties. Several N-acyl derivatives composed of conjugates between amino acids and fatty acids were synthesized by condensing D-tryptophan, D-alanine, D-methionine, D-valine, and D-aspartic acid with succiminidyl sorbate, myristate, palmitate, or caproate. Sorboyl-tryptophan, sorboyl-D-alanine, myristoyl-D-aspartic acid, and glycol-D-alanine strongly inhibited *Clostridium botulinum* when combined with 0.006% sodium nitrite (Paquet and Rayman, 1987), but it seems that these compounds have not been investigated further as food antimicrobials.

Several lipopeptides including polymyxin and lipopeptide antibiotics have potent antimicrobial activity and could be effective food preservatives. Although many are synthesized by food related bacteria such as *Bacillus* spp., they are considered antibiotics, and it is unlikely that they will see extensive use in food systems. The polymyxins consist of a fatty acid moiety covalently linked to a cyclic peptide. In contrast to many other lipophilic antimicrobials, the polymyxins have a strong preference for gram-negative bac-

teria. They cause direct membrane damage through a detergent action but at much lower concentrations than ordinary detergents. Membrane damage can be recognized by leakage of solutes, including nucleotides and inorganic ions, or by penetration of normally excluded molecules into the cell. Polymyxin is unique among related lytic agents in being bactericidal in the absence of cell growth (Davis, 1990). Polymyxins are useful as selective cleansers of infected intestinal tracts—as in the control of *Salmonella enteritidis* infections in poultry (Goodnough and Johnson, 1991). Lipopeptides synthesized by pseudomonads and bacilli have antimicrobial activity by virtue of their surfactant-like action (Kakinuma and Arima, 1969; Neu and Poralla, 1990; Vater, 1986).

Antimicrobial Plant Substances

Introduction

Compounds exhibiting various levels of antimicrobial activity are present naturally in plant stems, leaves, barks, flowers, and fruits. Information on the antimicrobial activity of plant substances and extracts has been available since the nineteenth century, but interest in naturally occurring antimicrobials declined during the first half of the twentieth century, possibly because of the development of highly effective synthetic antimicrobials (Delaquis and Mazza, 1995).

The compounds responsible for the desirability of some of the flavors and aromas of foods also are inhibitory to microorganisms. Spices and herbs, for example, have been used for millennia to provide distinctive flavors to foods and beverages (Beuchat, 1994). In many instances, however, the concentrations of compounds in spices and herbs necessary for inhibiting microorganisms exceed those resulting from normal usage levels in foods (Beuchat, 1994; Shelef, 1984).

Plants have developed mechanisms for defense against invasion by bacterial, fungal, or herbivorous insect and animal enemies (Walker, 1994). It is believed, however, that as humans have bred plants for nutrition, varieties or cultivars with decreased amounts of the unpleasant-tasting chemicals that are part of their defense mechanisms have resulted. Thus, domesticated plants may be more susceptible to disease than wild ones. They do, however, still contain at least some inducible chemical defense activity. Plant derived antimicrobial agents such as spices and essential oils have been used for their flavoring and antimicrobial properties for many centuries. For ex-

ample, the use of hops in beer-making was introduced in the Middle Ages to extend product shelf-life. Antimicrobial compounds present in food plants, as compiled and reviewed by Walker (1994), appear in Table 3.1.

A compound involved in plant defense mechanisms may be classified as a preinfectious or a postinfectious factor, as follows: (1) *prohibitins*, which are metabolites; (2) *inhibitins*, which are metabolites requiring a postinfectious increase in concentration for expression of full activity; (3) *postinhibitins*, which are toxic metabolites formed after infection through hydrolysis or oxidation of precursor compounds; and (4) *phytoalexins*, which are antimicrobial compounds formed *de novo* only after invasion of the host plant (Walker, 1994).

The *secondary metabolites*, or defensive antimicrobials produced by plants, are formed through one or more of five biosynthetic pathways: (1) as derivatives of sugar metabolism such as inositols and amino sugars found in antibiotics, e.g., glycosides; (2) through the acetate-malonate pathway forming fatty acid derivatives, e.g., polyacetylenes and polyketides; (3) through the acetate-mevalonate pathway leading to formation of terpenoids and steroids; (4) through the shikimic acid pathway forming phenylalanine and leading to formation of many aromatic and phenolic compounds; and (5) as metabolites derived from amino acids, e.g., alkaloids and nonprotein amino acids (Walker, 1994).

According to Walker (1994), the prohibitins include phenolic compounds (e.g., catechol, protocatechuic acid, 3,4-dihydroxyphenylalanine [DOPA], caffeic acid, chlorogenic acid, catechin, cyanidin, scopoletin, and juglone) (Figure 3.10), methylated flavones (e.g., tangeretin, nobiletin, and sakuranetin), flavonols (e.g., morin, maringenin, and hesperitin), hydroxyphenyl-threne derivatives (e.g., hircinol and isobatasin), compounds containing *p*-coumaroyl residues linked to arginine (e.g., hordatines A and B), glucosides (e.g., dihydroxymethoxybenzoxazinone-[DIMBOA]), glycosides (e.g., avenacins), alkaloids (e.g., α -tomatine), long-chain alcohols (e.g., 1,2,3-trihydroxyheptadec-16-ene), dienes (e.g., 1-acetoxy-2-hydroxy-4-oxoheneicos-12,15-diene), lactones (e.g., borbonol), polyacetylenes (e.g., wyerone, wyerone acid, falcarindiol, falcarinol, and passicol), and protein-like compounds (e.g., thaumatin-like proteins and zeamatin).

Sources of these compounds include potatoes for scopoletin; citrus plants for tangeretin, nobiletin, monin, naringenin, and hesperitin; black currants for sakuranetin; yams for isobatasin; barley seedlings for hordatines A and B; wheat and maize for DIM-

BOA; oat roots for avenacins; barley grain for thaumatin-like proteins; maize grain for zeamatin; potato skins for alkaloids; tomatoes for α -tomatine; avocado fruit for 1,2,3-trihydroxy heptadec-16-ene, borbonol, and 1-acetoxy-2-hydroxy-4-oxoheneicos-12; beans for wyerone and wyerone acid; and carrot roots and tomatoes for falcarindiol, falcarinol, and passicol (Walker, 1994).

As indicated, certain compounds, phenolic or flavonoid in nature, exert activity as a response to invasion. This activity usually is associated with the action of the enzyme diphenol oxidase. Many plants contain phenolic compounds in the form of hydrolyzable tannins, i.e., gallotannins and ellagitannins, which have extensive abilities to denature protein. This activity causes an astringent taste and antimicrobial activity in plant extracts (Walker, 1994).

The postinfectious defensive agents of many plants may be stored as inactive precursors activated when needed to fight invasion. Activation is catalyzed by hydrolases or oxidases, which are released by the host plant or by the invading fungus. Examples are the sulfoxides of onion and garlic. In garlic, the precursor alliin is degraded by the enzyme alliinase to yield allicin. Many plants contain cyanogenic glycosides hydrolyzed by specific α -glycosidases to release HCN, a microbial inhibitor in plants such as sorghum and lima beans and toxic to animals (Walk-

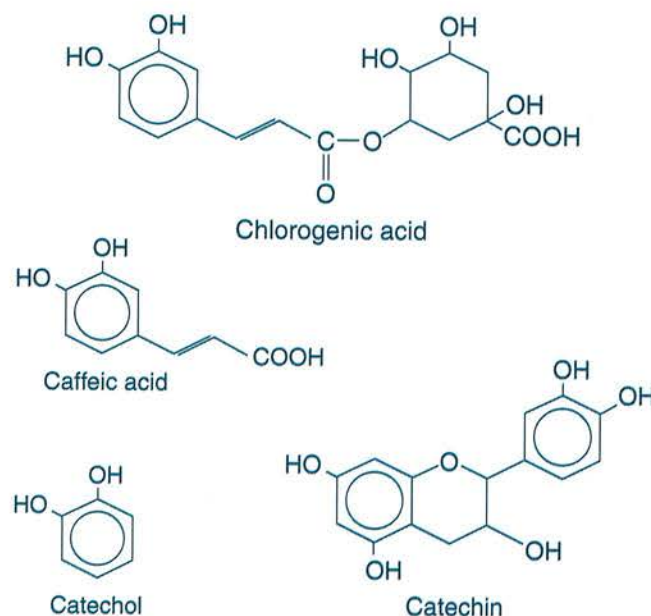


Figure 3.10. Plant phenolic compounds that are associated with antimicrobial activity.

Table 3.1. Antimicrobials found in food plants (from Walker, 1994)

Plant family	Common name	Plant species	Antimicrobials	Chemical type	Activity
<i>Amaryllidaceae</i>	Garlic	<i>Allium sativum</i>	Allicin	Allyl sulfoxides	Antibacterial/ antifungal
	Onion	<i>Allium cepa</i>	Protocatechoic acid	Phenolic acids	Antifungal
<i>Anacardiaceae</i>	Cashew nut	<i>Anacardium occidentale</i>	Anacardic acid	Substituted resorcinols	Antibacterial
	Mango	<i>Mangifera indica</i>			Antifungal
<i>Cannabinaceae</i>	Hops	<i>Humulus lupulus</i>	Humulone, lupulone	Prenylated polyketide	Antibacterial
<i>Chenopodiaceae</i>	Red beet	<i>Beta vulgaris</i>	Betavulgarin ^a	Isoflavone ^a	
<i>Compositae</i> (<i>Asteraceae</i>)	Safflower	<i>Carthamus tinctorius</i>	Safynol ^a	Polyacetylene	Antifungal
<i>Convolvulaceae</i>	Sweet potato	<i>Ipomoea batatas</i>	Ipomeamarone ^a	Furanoterpene	Antifungal
<i>Cruciferae</i> (<i>Brassicaceae</i>)	Cabbage	<i>Brassica oleracea</i>	Rapine, sinigrin	Glucosinolates	Antifungal
	Radish	<i>Raphanus sativus</i>	Raphanin		Antibacterial
	Turnip	<i>Brassica rapa</i>	Rapine		Antifungal
<i>Discoreaceae</i>	Yam	<i>Discorea rotundata</i>	Hircinol ^a , isobatatasin ^a	Phenanthrenes	
<i>Gramineae</i> (<i>Poaceae</i>)	Barley	<i>Hordeum vulgare</i>	Hordatin ^a		Antibacterial/ antifungal
	Maize	<i>Zea mays</i>	Thaumatococin-like proteins	Protein	Antifungal
	Oats	<i>Avena sativa</i>	Zeamatin	Protein	Antifungal
	Rice	<i>Oryza sativa</i>	Avenacins ^a , avenalumin ^a	Benzoxazin-4-one	Antifungal
			Momililactones ^a	Diterpene	Antifungal
			Oryzalexin ^a		
<i>Juglandaceae</i>	Walnut	<i>Juglans nigra</i>	Juglone	Phenolic	Antibacterial/ antifungal
<i>Lauraceae</i>	Avocado	<i>Persea</i> spp.	Borbonol ^a	Polyacetylenes	Antifungal
<i>Leguminosae</i> (<i>Fabaceae</i>)	Broad bean	<i>Vicia faba</i>	Wyerone acid ^a	Furanoacetylene	Antifungal
	Chick pea	<i>Cicer arietinum</i>	Medicarpin ^a	Pterocarpan	Antifungal
	French bean	<i>Phaseolus vulgaris</i>	Phaseollin ^a	Pterocarpan	Antifungal
	Pea (shoot)	<i>Pisum sativa</i>	Pisatin ^a	Pterocarpan	Antifungal
	Peanut	<i>Arachis hypogaea</i>	Resveratrol ^a	Stilbene	Antifungal
	Pigeon pea	<i>Cajanus cajan</i>	Stilbene-2-carboxylic acid		
			Glyceollin ^a		
	Soybean	<i>Glycine max</i>		Pterocarpan	Antifungal
<i>Moraceae</i>	Mulberry	<i>Morus alba</i>	Mulberrofuran D, albafuran A, D	Benzofuran	Antifungal
			Moracin M ^a		
<i>Oleaceae</i>	Olive	<i>Olea europaea</i>	Oleuropein	Phenolic glycoside	Antibacterial
<i>Passifloraceae</i>	Banana, passionfruit	<i>Passiflora mollisima</i>	Passicol	Polyacetylene	Antifungal
<i>Rosaceae</i>	Apple	<i>Malus</i> spp.	Phloretin, hydroxybenzoic acid, anthocyanidins	Phenolic glycosides	Antifungal
	Pear	<i>Pyrus</i> spp.	Arbutin	Phenolic glycoside	
<i>Rutaceae</i>	Citrus leaves	<i>Citrus</i> spp.	Nobiletin	Methylated flavones ^a	Antifungal
<i>Solanaceae</i>	Eggplant	<i>Solanum melongena</i>	Aubergenone ^a	Sesquiterpenes	Antifungal
	Green pepper	<i>Capsicum</i> spp.	Capsidiol ^a	Diterpene	Antifungal
	Potato	<i>Solanum tuberosum</i>	Rishitin ^a , Hydroxylobimin ^a , Caffeic acid, Scopoletin α -Tomatine	Phenolic, coumarin	
	Tomato	<i>Lycopersicon esculentum</i>		Alkaloid	Antifungal
<i>Umbelliferae</i>	Carrot (roots)	<i>Daucus carota</i>	Falcarindiol ^a	Polyacetylene	Antifungal
			6-Methoxymellein ^a	Dihydroisocoumarin	Antibacterial
<i>Vitaceae</i>	Grape	<i>Vitis</i> spp.	Viniferin ^a	Stilbene	

^aPhytoalexins (synthesized primarily after infection).

er, 1994).

Isothiocyanates (Table 3.2) are derived from the glycosides glucosinolates, which are stored in cell vacuoles of plants in the family Cruciferae (Delaquis and Mazza, 1995) and may have potential applications as vapors in food preservation (Isshiki et al., 1992). When plant tissues are injured or their integrity disrupted, glucosinolates are hydrolyzed rapidly by the enzyme myrosinase (thioglucoside glucohydrolase) to produce thiocyanates, isothiocyanates, nitriles, and glucose (Figure 3.11). Sometimes these products are unstable and decompose quickly to form a variety of other components (Delaquis and Mazza, 1995; Musk and

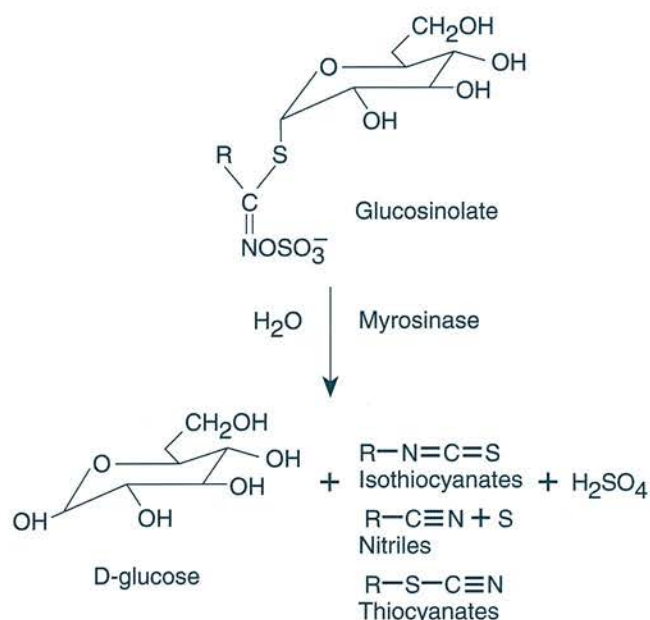


Figure 3.11. Isothiocyanates are stored in the cell vacuoles of plants in the family Cruciferae and have antimicrobial properties (from Delaquis and Mazza, 1995).

Johnson, 1993). The type of isothiocyanate formed depends on the R-side group of the original glucosinolate, which in turn depends on the plant source (Table 3.2). Glucosinolates and their hydrolytic products are found in plants such as cabbage, Brussels sprouts, cauliflower, broccoli, kale, kohlrabi, turnips, rutabagas, mustard, and rapeseed (Figure 3.12). Mustard oil was used by the ancient Romans to preserve fruit juices and wines. Isothiocyanates are believed to exert inhibitory activity against molds, yeasts, and bacteria, and it is thought that their antimicrobial activity is based on oxidative cleavage of the disulfide bonds, a process that may inactivate intracellular enzymes (Delaquis and Mazza, 1995). It also is believed that isothiocyanates, either as natural components or as added ingredients in the form of mustard or horseradish, contribute to food quality (Radford and Board, 1993; West et al., 1977).

In addition to having natural antimicrobial activi-



Figure 3.12. Cabbage is an example of a plant in the family Cruciferae that contains isothiocyanates.

Table 3.2. Isothiocyanate types, plant sources and their flavor/taste descriptors (modified from Delaquis and Mazza, 1995)

Isothiocyanate R-side group	Plant source	Flavor/taste descriptors
Allyl	Horseradish, mustard, turnip, cabbage, Brussels sprouts, kale, collards, cauliflower	Mustard, pungent, lachrymator
3-butenyl	Horseradish, mustard, cole crops, turnip, rutabaga	Horseradish, pungent
Benzyl	Cress, radish, horseradish, Nasturtium	
2-butyl	Horseradish, cabbage, Brussels sprouts, cauliflower, mustard, spinach	Acid, leaf green aroma
<i>p</i> -hydroxy-benzyl	Mustard, charlock	Pungent, no aroma
Methyl	Cabbage, cauliflower, Brussels sprouts, horseradish, radish	Horseradish, pungent, lachrymator
4-methylthio-3-butenyl	Radish	Pungent, sulfurous
4-pentenyl	Horseradish, mustard, turnip, rutabaga, cress, radish	Acid, fragrant leaf
2-phenylethyl	Horseradish, watercress, turnip, rapeseed	Horseradish, watercress,
Phenyl	Mustard, spinach, horseradish	turnip, tingling

ty by isolated phenolic compounds, diphenol oxidase enzymes (e.g., catecholases and laccases) act on phenolic compounds to yield products of increased antimicrobial activity. These enzymes, also involved in the defense mechanisms of insects, are present in almost all plants, where they oxidize dihydroxyphenols to form quinones, which are quite reactive and toxic. When the plant tissue and its membranes are damaged, the enzyme is released, and, as it comes in contact with the substrate, it forms the quinone inhibitors. These quinones can react with themselves or with proteins and amino acids of the plant, or of invading microorganisms forming dark, i.e., melanoid, pigment polymers, which are inhibitory to microorganisms. Common substrates for phenol oxidases include chlorogenic acid, catechin, epicatechin, and DOPA (Walker, 1994).

In addition to phenol oxidase, plant defense mechanisms are enhanced by the action of hydrolytic enzymes on phenolic compounds resulting in the formation of diphenol oxidase substrates, i.e., *aglycones*. This enhancement has been observed in apple and pear leaves, where phloridzin is hydrolyzed to phloretin, which is then oxidized to 3-hydroxyphloretin, the substrate of diphenol oxidase. In green olives, oleuropein (see following discussion) is hydrolyzed to aglycone elenolic acids that are inhibitory against microorganisms such as *Lactobacillus* spp. (Walker, 1994).

Plant pigments, organic acids, and several other phenolic compounds in plant materials also exhibit antimicrobial activity, as do phytoalexins produced in response to microbial infection, plant tissue injury, or naturally occurring elicitors. For extensive information on naturally occurring plant substances, the reader is referred to reviews by Marth (1966), Banks et al. (1986), Beuchat and Golden (1989), Wilkins and Board (1989), Conner (1993), Beuchat (1994), Lattanzio et al. (1994), and Walker (1994).

Garlic and Onion

Plants in the *Allium* genus, namely garlic (*A. sativum*) (Figure 3.13), onion (*A. cepa*), and leek (*A. porrum*), are probably the most widely consumed foods with substantial antimicrobial activity. Garlic has been used for medicinal purposes for centuries. It was not until the 1940s, however, that scientific evidence confirmed that garlic does indeed possess antimicrobial and medicinal properties. Cavallito and Bailey (1944) and Cavallito et al. (1945) isolated the major antimicrobial component from garlic bulbs. By using steam distillation of ethanolic extracts, they identified

this component as allicin, a diallyl thiosulfinate, i.e., 2-propenyl-2-propenethiol sulfonate, and described it as an extremely pungent, colorless oil responsible for the principal odor and taste of garlic and onion. At concentrations of 1:85,000 in laboratory culture broth, allicin was bactericidal to a wide range of gram-negative and gram-positive organisms.

Intact tissues of garlic and other *Allium* species do not contain allicin but do contain the precursor, alliin (S-allyl-L-cysteine-S-oxide). When bulb tissue is disrupted, alliin undergoes hydrolysis to yield allicin (Figure 3.14), pyruvate, and ammonia by the action of the phosphopyridoxal enzyme alliinase (Stoll and Seebeck, 1949). The mechanism of antimicrobial activity of garlic is inhibition of enzyme activity, including alkaline phosphatase, invertase, urease, and papain (Wills, 1956). Allicin has been shown to inhibit sulfhydryl enzymes but very few nonsulfhydryl enzymes. Sulfhydryl enzymes can be protected by cysteine or glutathione against inhibition by allicin, but only partial recovery of the enzyme activity is obtainable after the allicin and enzyme have been in contact for a short period.

Wills (1956) concluded that inhibition of sulfhydryl enzymes was associated with the presence of the -S-O-S- chemical grouping and not of the -S-O-, -S-S-, or -S- groups. Most sulfhydryl enzymes were inhibited by a 0.0005 molar (M) concentration of allicin. These included succinic dehydrogenase, urease, papain, xanthine oxidase, choline esterase, hexokinase, choline oxidase, glyoxalase, triose phosphate dehydrogenase, and alcohol dehydrogenase. Other sulfhydryl enzymes, e.g., carboxylases, adenosine triphosphatase, and β -amylase, were unaffected by 0.0005 M



Figure 3.13. Garlic, a plant in the *Allium* genus (*Allium sativum*), as well as onion and leek are probably the most widely consumed foods with substantial antimicrobial activity.

allicin. Among the nonsulfhydryl enzymes inhibited were lactic dehydrogenase, tyrosinase, and alkaline phosphatase. Ajoene is a garlic derived, sulfur-containing compound that exerts broad-spectrum antimicrobial activity (Naganawa et al., 1996).

Much of the research on the antimicrobial activity of garlic and other *Allium* species has been conducted by using foodborne pathogenic bacteria, mycotoxigenic molds, and spoilage microorganisms. Because sulfhydryl enzymes are common to all these microorganisms, the spectrum of activity of *Allium* extracts is broad. Some early studies showed that allicin, at a dilution of 1:125,000, prevented growth of *Staphylococcus aureus* and *Bacillus* spp. (Cavallito and Bailey, 1944). Mantis et al. (1978) reported that a 5% garlic extract solution had a germicidal effect whereas concentrations of garlic extract equal to or greater than 2% were inhibitory, and concentrations less than 1% were not. In general, garlic extract was more inhibitory at 37°C and pH 7.4 than at 28°C and pH 6.0. The authors concluded that sausage containing greater than 1% garlic was not a favorable environment for growth of *S. aureus*, especially after the pH decreased as a result of fermentation. Tynecka and Gos (1973) observed that an aqueous dilution of 1:256 garlic juice was inhibitory to *S. aureus*, whereas a cidal effect was exhibited by a 1:32 dilution. Cavallito and Bailey (1944) reported that garlic extract inhibited growth of *Streptococcus* at a much lower concentration (1:125,000 dilution) in a laboratory culture medium.

Aqueous extracts from fresh garlic bulbs at concentrations of 3, 5, and 10% inhibited growth of *Bacillus cereus* on nutrient agar plates by 31.3, 58.2, and 100%, respectively (Saleem and Al-Delaimy, 1982). Extracts

from garlic bulbs stored frozen at -18°C were slightly more inhibitory than extracts from bulbs stored at 15 to 35°C for six months. Gamma-irradiation of bulbs at a dose of 0.47 kilogray, with subsequent freezing before extraction, decreased original activity by as much as half. Exposing extracts to heat treatments of 80 to 90°C for 5 min completely destroyed activity against *B. cereus*. An investigation of the effect of garlic and onion oils on toxin production by *Clostridium botulinum* type A in meat slurry indicated that 0.15% inhibited but did not prevent toxin production at 20°C (DeWit et al., 1979). Toxin production by *C. botulinum* types B and E was not inhibited. The authors concluded that garlic and onion oils should not be applied in the meat industry for the purpose of inhibiting toxin production because of their evident ineffectiveness against nontype A *C. botulinum*.

Karaioannoglou et al. (1977) studied the effect of garlic extract on lactic acid bacteria. Growth of *Lactobacillus plantarum* in laboratory culture broth containing greater than 1% extract was inhibited. Growth inhibition at 30°C and pH 6.6 was less than that at 37°C and pH 7.4. Garlic extract concentrations greater than 1% were inhibitory to *L. plantarum* whereas lower concentrations, under favorable pH and temperature conditions, could be considered less inhibitory and might permit growth if large initial microbial populations (> 10⁶ cells/mL) were present.

Like gram-positive bacteria, a large number of gram-negative bacteria are inhibited by extracts from *Allium* species. Srivastava et al. (1982) compared fresh garlic extract to the antibiotic ampicillin for activity against 21 strains of gram-negative bacteria. These included species of *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, and *Shigella*. Inhibition by the extract was more effective than 0.003% ampicillin for inhibiting all test organisms except *Salmonella arizonae* and *Shigella cloacae*. The inhibitory effect of boiled extract was similar to that of unheated extract. Upon storage, however, the antibacterial properties of garlic extract diminished.

Johnson and Vaughn (1969) observed bactericidal activity of freshly reconstituted, dehydrated onion and garlic at concentrations of 1 and 5% (w/v), respectively, against *Salmonella typhimurium* and *Escherichia coli*, whereas maximal death rates occurred with concentrations of 5 and 10%. At the higher concentrations, decimal reduction times at 37°C were 1.1 and 1.2 hours, respectively, for resting *S. typhimurium* cells and 1.8 and 2.1 hours, respectively, for growing cells. Of the major volatile aliphatic disulfide compounds in onions, n-propyl allyl and di-n-propylallyl,

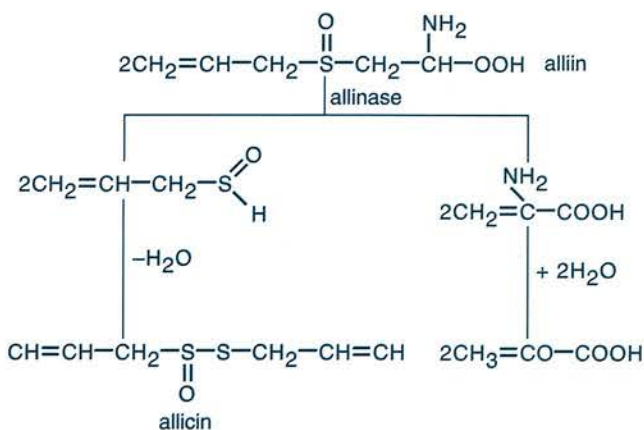


Figure 3.14. Garlic and other *Allium* species contain alliin, a precursor of allicin, which is released when bulb tissue is disrupted.

at concentrations of 0.1%, showed similar activity against resting cells. At similar concentrations, growing cultures of *E. coli* were more susceptible than *S. typhimurium* to garlic but evidently more resistant to onion.

Tynecka and Gos (1973) reported that growth of *Escherichia coli* was inhibited by a 1:128 dilution of garlic juice and inactivated by a 1:64 dilution. Crude juices and solvent extracts of garlic, onion, and *Allium kurrat* were reported by Abdou et al. (1972) to prevent growth of *E. coli*, *Pseudomonas pyocyaneus*, *Salmonella typhimurium*, and *Bacillus cereus*. Inhibition by crude juices seemed greater than inhibition by ether, chloroform, or ethanol extracts. The researchers concluded that both garlic and onion were strong antiseptics and could be used for medicinal applications.

A large number of yeasts and molds are susceptible to the inhibitory action of *Allium* extracts. Tynecka and Gos (1973) reported that garlic juice was very potent against *Candida albicans* and speculated that fungal skin diseases could be treated with garlic extract. The yeast was inhibited by a 1:512 dilution of juice and inactivated by a 1:256 dilution. Barone and Tansey (1977) attributed inhibition of 39 out of 41 strains of *C. albicans* by aqueous extracts of garlic to destruction of thiols such as L-cysteine and glutathione. The researchers hypothesized that additional *cidal*—i.e., lethal, or *static*—i.e., inhibitory, effects of allicin are due to its ability both to inactivate proteins by oxidation of essential thiols to the disulfide and to inhibit completely the activity of these sulfhydryl compounds competitively, by combining with them. Activity was unaffected at pH 2.0 to 6.0, decreased at pH 9.0, and eliminated at pH 12.0. Extract heated at 121°C for 10 min retained no anticandidal activity.

Growth of other yeasts also is inhibited by extracts of *Allium* spp. Moore and Atkins (1977) reported that a variety of yeast-like fungi representing the genera *Cryptococcus*, *Rhodotorula*, *Trichosporon*, and *Torulopsis*, were inhibited *in vitro* in the presence of an aqueous extract of garlic at concentrations diluted as much as 1:1024 in water. In addition, the researchers observed that 22 actively pathogenic isolates of *Candida albicans* were inhibited by the garlic extract.

An investigation of the inhibitory effects of 32 essential oils from plants on 13 food spoilage yeasts was conducted by Conner and Beuchat (1984a). At concentrations as low as 0.0025%, garlic oil was a potent inhibitor of growth. Onion oil also was strongly inhibitory. Resuscitation of heat stressed yeasts representing several food spoilage genera (*Candida*

lipolytica, *Debaryomyces hansenii*, *Hansenula anomala*, *Kloeckera apiculata*, *Lodderomyces elongisporus*, *Rhodotorula rubra*, *Saccharomyces cerevisiae*, and *Torulopsis glabrata*) was less inhibited by garlic oil than by onion oil (Conner and Beuchat, 1984b, 1985). Essential oil and oleoresin of garlic adversely affected ethanol production by *S. cerevisiae* (Conner et al., 1984). Sporulation of *H. anomala* and *L. elongisporus* was delayed by garlic oil.

Growth of aflatoxin-producing molds, namely *Aspergillus flavus* and *A. parasiticus*, was inhibited by various extractives of onion, including ether extracts, as well as by lachrymatory factor, i.e., thiopropanal-S-oxide, and steam distilled onion oil (Sharma et al., 1979). Ethyl acetate was ineffective. Exposure of onions to gamma-irradiation with a sprout-inhibiting dose (0.06 kilogray) did not alter the inhibitory potency of the onion extractives, which, however, seemed heat labile. Sharma and colleagues (1981a) reported later that germinated conidia were more susceptible to inhibition by onion extracts than ungerminated conidia were. The *cidal* effect of extract against conidia of *A. parasiticus* was lost by heating, freeze-drying, dehydrating, aerating, and storing for prolonged periods.

Mabrouk and El-Shayeb (1981) studied the effect of garlic on mycelial growth and aflatoxin production by *Aspergillus flavus*. Minced garlic was more effective than garlic extract in inhibiting aflatoxin formation. Heat treatment at 121°C decreased the potency of minced garlic, but by using a freeze-drying technique, Sharma et al. (1981b) demonstrated that sporicidal activity of onion extracts against *A. parasiticus* could be retained. Garlic and onion extracts inhibit the growth of many other molds, some of which are known to spoil grains, legumes, and processed foods (Appleton and Tansey, 1975; Coley-Smith and King, 1969; Tansey and Appleton, 1975). Even when growing in soil, garlic and onion can influence the microflora in the rhizosphere. Timonin and Thexton (1950), for example, observed that rhizosphere soil in proximity to garlic and onion harbored 11 and 12 times more bacteria and 6 and 13 times more actinomycetes, respectively, than control soil did. The microfloral profile in soil adhering to garlic and onion bulbs and roots as they were taken from the field for processing therefore may be significantly different from that in soil adhering to the roots of other crops.

Fliermans (1973) demonstrated inhibition of *Histioplasma capsulatum* by extracts of the garlic plant. The mycelial phase of the organism was inhibited by both volatile and water-soluble components of garlic at concentrations of 0.0000254%. The problem of *H.*

capsulatum infectivity is most significant in soils exposed to bird excreta, a situation common in soils adjacent to bird roosting sites. Although laboratory data were not extrapolated to the field, application of the active component of garlic to infected soils may inhibit the organism. Tansey and Appleton (1975) also reported that garlic extract inhibited the growth of *H. capsulatum*.

The lethal activities of garlic against mosquitoes (*Culex pipiens quinquefasciatus*) were studied by Amonkar and Banerji (1971). Diallyl disulfide and diallyl trisulfide, alone or combined at a concentration of 0.0005%, were larvicidal whereas 0.02% of diallyl sulfide and dipropyl disulfide were ineffective. The authors also observed antagonistic properties of diallyl di- and trisulfide against several other pests of economic importance such as the potato tuber moth, the red cotton bug, the red palm weevil, and the housefly.

Phenolic Compounds in Spices and Herbs

In addition to contributing to the sensory quality of foods, many spices and herbs also exhibit antimicrobial activity. In spices and herbs, the compounds largely responsible for antimicrobial activity include many simple and complex derivatives of phenol that are volatile at room temperature. In many instances, concentrations of these compounds necessary for inhibiting growth or various metabolic activities in microorganisms exceed those normally used in foods. Nevertheless, the preservative effects of such seasoning agents should not be discounted.

Among the spices with the greatest antimicrobial



Figure 3.15. Cinnamon, which inhibits bacterial activity, mold growth, and mycotoxin production, is being stripped from the tree. Photograph courtesy of Larry R. Beuchat, University of Georgia, Griffin.

activity are cinnamon (Figures 3.15 and 3.16), clove, and allspice. Their antimicrobial principle(s) often is(are) present in the *essential oil*, or in the extracted, isolated, and concentrated natural oil, of these spices. Cinnamic aldehyde (Figure 3.17), i.e., 3-phenyl-2-propenal, the major antimicrobial compound in cinnamon not only exhibits antibacterial activity (Deans and Richie, 1987) but also inhibits mold growth and mycotoxin production. Hitokoto et al. (1978) reported that cinnamon bark had a strong inhibitory effect on fungi, including *Aspergillus para-*



Figure 3.16. Rolls of cinnamon being dried. Photograph courtesy of Larry R. Beuchat, University of Georgia, Griffin.

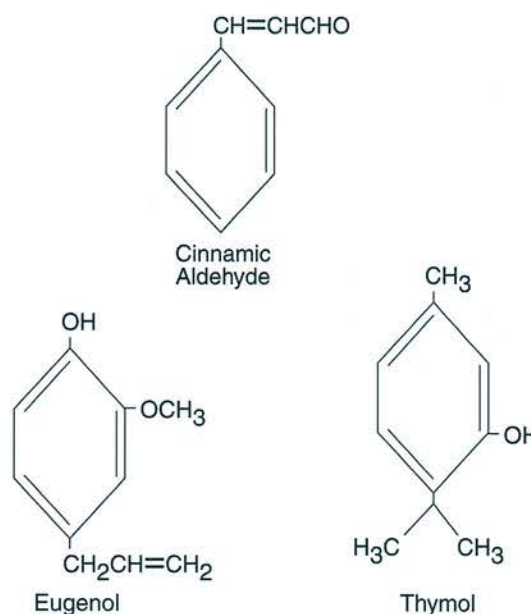


Figure 3.17. Cinnamic aldehyde, thymol, and eugenol structures—antimicrobial compounds in spices.

siticus. Bullerman (1974), who also observed the inhibitory effect of cinnamon on *A. parasiticus*, reported that a 1 to 2% concentration of ground cinnamon in broth would allow some growth of *A. parasiticus* but would decrease aflatoxin production by 99%. In a later study, Bullerman et al. (1977) demonstrated that the essential oil of cinnamon at a concentration of 0.02% was inhibitory to growth and subsequent toxin production by *A. parasiticus* and that cinnamic aldehyde was inhibitory at a concentration of 0.015%. Other unsaturated aldehydes (citral and citronellol), an unsaturated alcohol (geraniol), and a terpene alcohol (menthol) also have exhibited various degrees of antimycotic activity (Moleyar and Narasimham, 1986). Geraniol and citronellol also have inhibited the growth of *Erwinia* bacteria (Scortichini and Rossi, 1991).

Eugenol (Figure 3.17), i.e., 2-methoxy-4-(2-propenyl)phenol, a major constituent in clove oil and present in considerable amounts in the essential oil of allspice, possesses antimicrobial activity. Karapinar and Aktug (1987) reported that eugenol was more effective against *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus* than thymol, anethol, or menthol was. Clove powder (0.12%) and eugenol (0.02%) adversely affect germination rate of *Bacillus subtilis* spores (Al-Khayat and Blank, 1985; Blank et al., 1987). Spores of the same bacterium had increased sensitivity when conditioned in clove powder (64 to 98% relative humidity, 25 to 35°C) before heat treatment (Blank et al., 1988). Bullerman et al. (1977) demonstrated that clove oil at 0.025% inhibited growth and toxin production by *Aspergillus parasiticus* and that eugenol was inhibitory at a concentration of 0.0125%. Hitokoto et al. (1980) observed that ground cloves incorporated into culture media completely inhibited growth of toxigenic *Aspergillus* spp.

The essential oils and *oleoresins*, i.e., extracts composed of oil-holding resin in solution, of cinnamon, clove, and allspice are known to inhibit the growth of several food spoilage and fermentation yeasts (Conner and Beuchat, 1984a). Resuscitation of heat stressed yeast cells was impaired in media containing as little as 0.0025% essential oil of cinnamon (Conner and Beuchat, 1984b, 1985). Vanillin (4-hydroxy-3-methoxybenzaldehyde), a major constituent in vanilla beans, is structurally similar to eugenol and also is antimycotic. The compound inhibits or retards the growth of yeasts (Boonchird and Flegel, 1982) and molds (Maruzzella and Liguori, 1958).

Thymol (Figure 3.17) (5-methyl-2-[1-methylethyl]phenol) present in the essential oils of thyme, orega-

no, savory, sage (Figure 3.18), and several other herbs is among the compounds with a wide spectrum of antimicrobial activity. Beuchat (1976) reported that essential oils of thyme and oregano were inhibitory to *Vibrio parahaemolyticus*. Of 50 plant essential oils examined by Deans and Ritchie (1987), thyme oil was the most inhibitory against 25 genera of bacteria. When used at a concentration of 0.05%, alcoholic extracts of these spices as well as those of rosemary and turmeric inhibited germination, growth, and toxin production by *Clostridium botulinum* (Huhtanen, 1980). The presence of 0.05% ethanolic extract of thyme inhibited growth of *Staphylococcus aureus* (Aktug and Karapinar, 1986); growth of *V. parahaemolyticus* was inhibited by 0.1, 0.5, and 0.6% of powdered thyme, bay leaf, and mint (Figure 3.19), respectively. Sage was inhibitory to *V. parahaemolyticus* (Shelef et al., 1980), *Bacillus cereus*, *S. aureus*, and *Salmonella typhimurium* (Shelef et al., 1984). Thymol also has been shown to inhibit growth and toxin production by mycotoxigenic molds (Akgul and Kivanc, 1988; Benjilali et al., 1984; Ray and Bullerman, 1982). Thymol concentrations equal to or greater than 0.05% completely inhibited growth of *Aspergillus parasiticus* whereas lower thymol concentrations caused partial or transitory growth and toxin production patterns (Buchanan and Shepherd, 1981). Hitokoto et al. (1980) also observed a strong antifungal activity of thymol. These researchers reported that a 2% concentration of oregano in potato dextrose agar medium completely inhibited the growth of seven mycotoxigenic molds. Maruzzella and Liguori (1958) reported that the volatile oils (essential oils) from origanum (oregano), savory, and thyme, when tested *in vitro* using a standard zone-of-inhibition test, possessed substan-



Figure 3.18. The herb, sage, which contains the antimicrobial thymol.

tial antifungal activities against 18 pathogenic and nonpathogenic fungi. Thyme and oregano actually can have a stimulatory effect on lactic acid production by *Lactobacillus plantarum* and *Pediococcus cerevisiae* used in meat fermentations (Zaika et al., 1983; Zaika and Kissinger, 1981). Manganese ions were identified as a factor in these and other spices responsible for the enhancement of acid production by meat fermentation starter culture bacteria (Zaika and Kissinger, 1984).

Farbood et al. (1976) reported that rosemary spice extract at 0.1% substantially inhibited growth of *Salmonella typhimurium* and *Staphylococcus aureus*. A concentration of 0.3% of either sage or rosemary in culture media inhibited the growth of 20 foodborne gram-positive bacteria whereas a concentration of 0.5% was bactericidal (Shelef et al., 1980). An investigation of the effect of turmeric on the growth of intestinal and pathogenic bacteria indicated that the oil fraction of turmeric inhibited numerous bacteria including *Bacillus cereus*, *S. aureus*, *Escherichia coli*,

and *Lactobacillus plantarum* (Bhavani Shankar and Screenivasa Murthy, 1979).

The mode of action of phenolic compounds in spices and herbs against microorganisms has not been defined clearly. Changes in membrane permeability and interference with enzyme function may be involved. Energy depletion in yeasts caused by allyl hydroxycinnamates (Baranowski et al., 1980) and essential oils of allspice, clove, cinnamon, oregano, thyme, and savory (Conner et al., 1984) has been reported. Ethanol production, respiratory activity, and yeast sporulation also can be influenced by essential oils of spices and herbs.

Still other plant parts and extracts used as spices and herbs are known to possess antimicrobial activity (Table 3.3). Foodborne pathogenic bacteria adversely affected by a wide range of compounds present in these seasoning agents include *Bacillus cereus*, *Clostridium botulinum*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*. Growth of the mycotoxigenic molds *Aspergillus flavus*, *A. parasiticus*, *A. versicolor*, *A. ochraceus*, *Penicillium urticae*, and *P. roquefortii* (Azzouz and Bullerman, 1982), as well as other food-spoilage molds, yeasts, and bacteria, also is retarded or inhibited in the presence of many spices and herbs commonly used as flavoring agents.



Figure 3.19. The herb, mint, which contains the antimicrobial thymol.

Plant Pigments and Related Compounds

Compounds responsible for color of plant tissues have, in many instances, antimicrobial properties. Anthocyanins, present in almost all higher plants and predominantly in flowers and fruits, consist of an aglycone—i.e., anthocyanidin—portion esterified with one or more sugars. All anthocyanidins are derivatives of the flavylum cation and have various degrees of hydroxylation and methoxylation. Pelargonidin, cy-

Table 3.3. Plants used as spices and herbs that also contain compounds possessing antimicrobial activity (adapted from Boonchird and Flegel, 1982; Conner and Beuchat, 1984a; Deans and Richie, 1987; Maruzzella and Liguori, 1958; Huhtanen, 1980; and Beuchat, 1994)

Achiote	Cananga	Coffee	Leek	Mustard	Peppermint	Tea
Allspice (pimenta)	Caraway	Coriander	Lemon	Nutmeg	Pimento	Thyme
Almond (bitter)	Cardamon	Dill	Lemongrass	Onion	Rosemary	Turmeric
Angelica	Celery	Elecampane	Licorice	Orange	Sage	Vanilla
Anise	Chenopodium	Fennel	Lime	Oregano	Sassafras	Verbena
Basil (sweet)	Cinnamon	Fenugreek	Mace	Paprika	Savory	Wintergreen
Bay (laurel)	Citronella	Garlic	Mandarin	Parsley	Spearmint	
Bergamot	Clove	Ginger	Marjoram	Pennyroyal	Star anise	
Calmus	Cocoa	Horseradish	Musky bugle	Pepper	Tarragon (estragon)	

anidin, delphinidin, petunidin, and malvidin are among the most important anthocyanidins in terms of their contributions to the sensory quality of foods.

Although anthocyanin pigments are better known for their food-coloring capabilities, they also inhibit bacteria. Hartman (1959) reported that pelargonidin 3-monoglucoside and its degradation products inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, and Powers et al. (1960) observed that certain anthocyanins inhibited *E. coli*, *S. aureus*, and *Lactobacillus casei*. Similar observations were made by Zimmerman (1959) with *L. acidophilus*. Pratt et al. (1960) showed that monoglucosides of cyanidin, pelargonidin, and delphinidin not only increased the lag phase of bacteria but also decreased the maximal growth attained. Flavanols and proanthocyanidins in cranberries are highly inhibitory to yeasts (Marwan and Nagel, 1986).

The mechanism of antimicrobial activity of anthocyanins is not understood fully. Carpenter et al. (1967) reported that anthocyanins had an inhibitory effect on certain bacterial enzymes. The chelating ability of anthocyanins described by Somaatmadja et al. (1964) may explain in part their inhibitory action on bacterial enzymes. Because the activity of many enzymes depends on metal ions, the unavailability of these metals could render the enzymes inactive and thus inhibit growth. The role of anthocyanins as chelators was substantiated by Somaatmadja et al. (1964), who reported that the addition of magnesium and calcium ions reversed bacterial inhibition caused by malvidin 3-monoglucoside.

Chlorophylls *a* and *b* are present in all higher plants. Upon degradation, chlorophyllides, phylophytins, pheaphorbides, and pyrrole compounds are formed. Chlorophyllide *a* is known to inhibit growth of *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* (Beuchat et al., 1966).

Other Plant Phenolic Compounds

Several phenolic compounds including oleuropein and its aglycone are found in olives (Figure 3.20) and olive oil (Table 3.4) (Dallyn, 1994; Fleming et al., 1969). The hydrolysis products of oleuropein, which contains glucose, β -3,4-dehydroxy-phenylethyl alcohol, and an acid, are β -3,4-dihydroxyphenylethyl alcohol, elenolic acid, and the oleuropein aglycons. Oleuropein may not be antimicrobial, but the aglycone and elenolic acid inhibit the growth of lactic acid bacteria (Fleming et al., 1973). Microorganisms inhibited by phenolic compounds present in olive oil include *Lactobacillus* spp., *Leuconostoc mesenteroides*, *Salmo-*

nella enteritidis, *Staphylococcus aureus*, and fungi, including *Geotrichum candidum*, *Rhizopus* spp., and *Rhizoctonia solani* (Dallyn, 1994; Juven and Henis, 1970). These compounds are considered responsible for occasional abnormal fermentation patterns in green olives. Degradation products of oleuropein are surface active and thus cause leakage of cytoplasmic constituents such as glutamate, potassium ions, and inorganic phosphate from bacterial cells (Juven et al., 1972). Aflatoxin production by *Aspergillus parasiticus* and *A. flavus* is decreased greatly in the presence of oleuropein (Gourama and Bullerman, 1987) and of ethanolic extracts of olive callus tissues (Paster et al., 1988). Decreased amount of aflatoxin produced is not correlated with decreased formation of mycelium.

Hydroxycinnamic and cinnamic acids are phenolic compounds present in plant parts used as spices (Beuchat and Golden, 1989). Compounds with antimicrobial activity include caffeic, chlorogenic, *p*-coumaric, ferulic, and quinic acids (Figure 3.10). Depending on the botanical species, hydroxy-cinnamic acids may be present at concentrations sufficient to retard microbial invasion and to delay rotting of fruits and vegetables. Gram-positive and gram-negative bacteria, molds, and yeasts commonly encountered as food-spoilage organisms are sensitive to hydroxycinnamic acid derivatives (Davidson and Branen, 1981). Caffeic, ferulic, and *p*-coumaric acids, for example, inhibit *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* (Herald and Davidson, 1983). Baranowski et al. (1980) reported that ferulic acid and, to a lesser extent, *p*-coumaric acid inhibited growth of *Saccharomyces cerevisiae*. These researchers suggested that naturally occurring hydroxycinnamic acids may inter-



Figure 3.20. Olives contain phenolic compounds that are antimicrobial.

ferre with the fermentation of fruit juices by this yeast. Caffeic acid and *o*-coumaric acid inhibited aflatoxin production by *Aspergillus flavus*, and coumarin inhibited fungal growth (Paster et al., 1988).

Various other phenolic compounds have been demonstrated to exhibit antimicrobial activity. Tannins and tannic acid are present in the barks, rinds, and other structural tissues of plants and are known to possess antimicrobial activity. Beuchat and Heaton (1975) attributed the toxic effect of pecan packing tissue on *Salmonella senftenberg* to a high concentration of tannins. Tannic acid was inhibitory towards *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Streptococcus faecalis* (Chung and Murdock, 1991). Tannic acid and tannins also exhibit fungistatic properties. Singleton and Esau (1969) predicted that the antimicrobial effect of red and white wines should be proportional to the amount of flavonoid tannin present.

The antiviral activity of extracts of blueberries,

crabapples, strawberries, red wines, grape juice, apple juice, and tea was studied by Konowalchuk and Speirs (1976a, b; 1978a, b). Some of these extracts inactivated poliovirus, Coxsackie virus, echovirus, reovirus, and herpes simplex virus. The researchers concluded that the primary inhibitors were tannins. Tannic acid was antiviral against echovirus, poliovirus, and herpes simplex virus. Cliver and Kostenbader (1979) also reported that grape juice was antiviral but that the effect was reversible.

Hops

Flowers of the hop, i.e., *Humulus lupulus*, vine (Figure 3.21) are used to impart bitter flavors and other desirable properties to beer. Resins, commonly termed α -acids, represented by humulone and its congeners—i.e., cohumulone, adhumulone, prehumulone, and posthumulone—and β -acids, represented by lupulone and its congeners—i.e., colupulone, adlupulone, prelupulone, and postlupulone—are the major compounds responsible for this flavor. Most of these compounds also inhibit microbial growth. Gram-positive bacteria and some fungi are most sensitive (Mizobuchi and Sato, 1985; Schmalreck et al., 1975). Undissociated molecules are mainly responsible for inhibition of *Lactobacillus brevis* (Simpson and Smith, 1992).

Lactobacillus spp. that may contaminate pitching yeasts develop resistance to humulone. Richards and Macrae (1964) reported that several strains of *Lactobacillus* spp. acquired an eightfold to twentyfold in-

Table 3.4. Phenolic antimicrobial compounds found in some olive oils and associated materials (from Dallyn, 1994)

Antimicrobial compound	Extra virgin olive oil	Rape of olives and tissue	Alpechines
Benzoic acid	+		
Caffeic acid	+	+	+
Catechol		+	
Chlorogenic acid			
Cinnamic acid	+	+	
<i>p</i> -Coumaric acid	+	+	
β -3,4-Dihydroxyphenylethanol	+		
3,4-Dihydroxyphenyl acetic acid	+		
Ferulic acid	+	+	+
Gallic acid	+		+
<i>p</i> -Hydroxybenzoic acid	+		+
<i>p</i> -Hydroxybenzaldehyde			+
<i>p</i> -Hydroxyphenyl acetic acid			+
<i>p</i> -Hydroxyphenyl propionic acid			+
Hydroxytyrosol	+		
Kinic acid		+	
Oleuropein	+	+	
Protocatechuic acid	+	+	
Protocatechuic aldehyde			+
Rutin	+		
Syringic acid	+	+	+
Syringaldehyde			+
3,4,5-Trimethoxybenzoic acid			+
Tyrosol	+	+	+
Vanillic acid	+	+	+
<i>p</i> -Vanillin			+
Veratric acid			+



Figure 3.21. Flowers of the hop vine, *Humulus lupulus*, are used to impart bitter flavors and antimicrobial properties to beer. Photograph courtesy of Larry R. Beuchat, University of Georgia, Griffin.

crease in resistance to 0.01% humulone after 2 to 4 subculturing steps. In the absence of humulone, resistant populations retain a degree of stability upon subculture. Hops therefore may contribute very little to the microbial stability of beer.

The antimycotic effect of hop extracts is influenced by water activity (Engelson et al., 1980). Germination of conidia and the rate of colony development by *Aspergillus niger*, *A. glaucus*, and a *Penicillium* sp. were affected adversely by extract amounts of approximately 1.5% in a laboratory medium. Effects were more pronounced as the water activity of the medium was decreased by addition of glycerol. The authors suggested that the combined effect of decreased water activity and hop extract may be used to impart biological stability to foods of intermediate moisture.

Coffee, Tea, Kola, and Cocoa

Caffeine (1,3,7-trimethylxanthine) is present in coffee and cocoa beans (Figure 3.22), tea, and kola nuts; it is antimycotic as well as antibacterial. Inhibition of growth of several mycotoxigenic *Aspergillus* and *Penicillium* species at concentrations of caffeine as low as 0.0001% has been documented (Buchanan et al., 1981, 1983a; Lenovich, 1981). Caffeine adversely affects the production of aflatoxin, ochratoxin A, sterigmatocystin, citrinin, and patulin.

The mechanism by which caffeine inhibits polyketide mycotoxin synthesis is unknown. Likewise, the mode of action on other metabolic functions of fungal cells has not been defined clearly. Tortora et



Figure 3.22. Caffeine, contained in coffee (above) and cocoa beans, is antimycotic as well as antibacterial. It also inhibits the production of mycotoxins. Photograph courtesy of Larry R. Beuchat, University of Georgia, Griffin.

al. (1982) reported that caffeine but not theophylline or papaverine uncoupled the regulation of glycolysis and glycogenesis in *Saccharomyces cerevisiae*. Inhibition of growth of *Aspergillus parasiticus* by caffeine is due in part to an alteration in purine metabolism, but inhibition of aflatoxin synthesis evidently does not involve inhibition of cyclic adenosine monophosphate phosphodiesterase or a chelation of key metal ions (Buchanan et al., 1983b). Caffeine may restrict glucose uptake by *A. parasiticus*, resulting in decreased aflatoxin production (Buchanan and Lewis, 1984). A preliminary examination of the effect of caffeine on patulin production by *Penicillium urticae* suggests that activity does not involve a generalized inhibition of lipid synthesis (Buchanan et al., 1983a).

Vanos and Bindschedler (1985) studied the effects of caffeine on *Lactobacillus* spp., the predominant group of microflora isolated from a commercial instant coffee processing facility. *Lactobacillus plantarum* was the dominant species. Total inhibition of growth was obtained at 0.0015% caffeine and at 60% total nondecaffeinated coffee solids. *Staphylococcus aureus*, a *Salmonella* serotype, *Escherichia coli*, *Streptococcus faecalis*, and *Bacillus cereus* failed to grow in 2% (w/v) reconstituted decaffeinated and nondecaffeinated coffee. The effect of caffeine on *Listeria monocytogenes* was investigated by Pearson and Marth (1990b). At a concentration of 0.5% in skim milk, growth at 30°C occurred although the lag phase was extended to 6 to 9 hours compared with less than 3 hours in milk not containing caffeine. Generation times were significantly longer in the presence than in the absence of caffeine (2.17 and 1.2 hours, respectively).

Tea and cocoa contain theophylline (i.e., 1,3-dimethylxanthine) and theobromine (i.e., 3,7-dimethylxanthine). Theophylline is present in manufactured tea at 0.00023 to 0.00044% whereas theobromine content is present at about 0.0005% (Lunder, 1979). Theobromine is the principal alkaloid in cocoa beans (1.5 to 3%) and also is present in kola nuts. Pearson and Marth (1990a) observed that adding cocoa to milk enhanced the growth of *Listeria monocytogenes*. In a related study (Pearson and Marth, 1990c), the pathogen was observed to have a longer lag phase in milk containing cocoa but reached a higher population than in milk without cocoa. The neutralization effect of casein on the antilisterial activity of cocoa (Pearson and Marth, 1990c) is in agreement with observations by Zapatka et al. (1977) on *Salmonella typhimurium*, who reported that adding 3 to 5% casein to 5% cocoa in distilled water resulted in 44 to 57% survival of the organism compared with no survival in the absence of casein. Neither theophylline nor theobromine seem

to have much effect on aflatoxin production by molds (Buchanan and Fletcher, 1978).

Growth of *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* were affected adversely in a 2% (total solids) instant tea infusion (Vanos et al., 1987). Inhibition of *Lactobacillus plantarum* was observed in a 10% tea infusion. It was concluded that a flavanol, i.e., catechin, plus caffeine complex was the only natural inhibitor of *Lactobacillus* spp. in instant tea. The linden flower, used to prepare tea in the Near East, has been reported to inhibit the growth of *S. aureus* and, to a lesser extent, *Salmonella typhimurium* and *Vibrio parahaemolyticus* (Gonul and Karapinar, 1987).

Carbohydrates

Perhaps the most widely occurring and abundant group of indirect natural antimicrobial compounds is the simple sugars occurring in foods of plant origin. The process of removing water from fruit and vegetable tissues, purees, and juices results in a proportional increase in the percentage of solid materials in these foods. As has been indicated, glucose, fructose, and other sugars, which have been used traditionally in foods, decrease the water activity of foods and thereby microbial spoilage. Carbohydrates also serve as nutrient substrates of microorganisms involved in fermentations resulting in generation of antimicrobial components. Other carbohydrates are converted to toxic metabolites, thereby killing or inhibiting their producers. *Streptococcus mutans* converts xylitol to xylitol-5-phosphate, which is lethal to the organism. Xylitol can cause inactivation of certain bacteria through this toxic metabolite (Scott, 1988).

Phytoalexins

Phytoalexins (Figure 3.23) are low molecular weight compounds produced by higher plants in response to microbial infection and naturally occurring elicitors (Dixon et al., 1983; Walker, 1994). The mechanism of action of phytoalexins, which are broad-spectrum antimicrobial agents, is not understood fully although evidence suggests that they alter properties of microbial plasma membranes. Production of phytoalexins by plant cell cultures was reviewed by Whitehead and Threlfall (1992) and by Walker (1994).

Production of phytoalexins is induced by trace amounts of elicitors produced by the invading microorganisms; their generation and action thus are dynamic processes occurring after tissue damage and invasion by phytopathogenic fungi. Their production

also is stimulated by other natural organic compounds, inorganic salts, and UV radiation (Mercier et al., 1993; Walker, 1994).

Pisatin, from peas (Figure 3.24), is the first recognized phytoalexin, but the total number of compounds isolated exceeds 200 from more than 20 families of plants, especially the *Leguminosae*. Other phytoalexins produced by this family are phaseollin (beans) and glyceollin (soybeans). Phytoalexins also are produced by sweet potatoes (ipomeamarone), potatoes (rishitin), sweet peppers (capsidiol), eggplants (auberginone), peanuts (resveratrol), grapes (viniferin), carrots (falcariindiol, 6-methoxymellein), and safflower (safynol) (Walker, 1994).

Because of their inhibitory effects on pathogenic and spoilage microorganisms, several phytoalexins are of particular interest to food microbiologists. Since 1940, these compounds have been known to occur in leaves, fruits, seeds, roots, and tubers of a wide range of plants (Table 3.5). Glyceollin, coumestrol, and glycinol, and all phytoalexins produced by soybeans inhibit microbial-membrane associated processes. The dominant antibacterial phytoalexin produced in soybeans inoculated with *Erwinia carotovora* and *Saccharomyces cerevisiae* is glycinol, which has been hypothesized to inhibit the growth of a wide range of

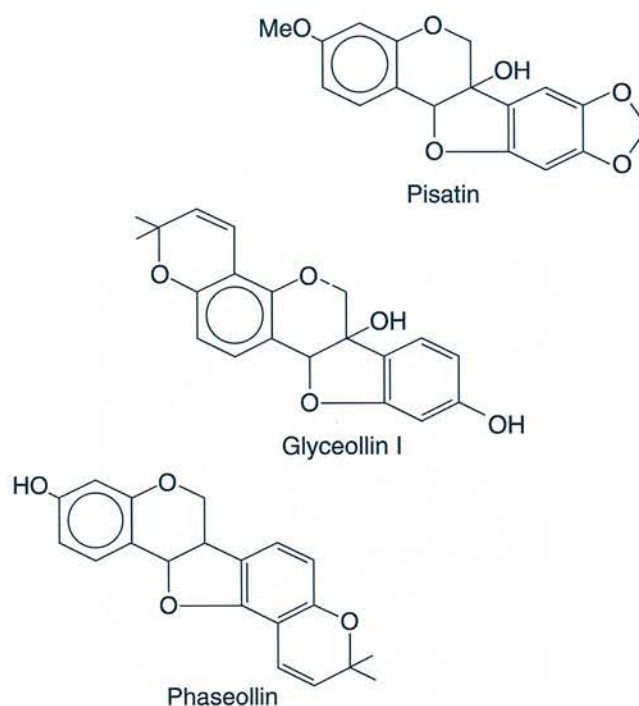


Figure 3.23. Phytoalexins isolated from plants in the family Leguminosae (from Walker, 1994).

other bacteria and fungi. Exposure of *Escherichia coli* membrane vesicles to glycinol resulted in inhibition of respiration linked transport (Weinstein and Albersheim, 1983). Numerous other phytoalexins are produced in soybeans and other legumes (Rizk and Wood, 1980). *Pseudomonas* and *Xanthomonas* species and several species of molds are affected adversely. Low molecular weight antimicrobial compounds also are known to be produced by green beans, broad beans, garden peas, cowpeas, and alfalfa. A broad range of fungi and bacteria are sensitive to these stress metabolites. Nonleguminous vegetables also can be induced to produce phytoalexins. Capsidiol from bell peppers (Stoessel et al., 1972), rishitin, lubimin, and phytuberin from potatoes and auberginone from eggplant (Dixon et al., 1983) all are known to be active against bacteria and fungi.

Dihydroisocoumarin, chromone, and scopoletin formation in carrot roots is induced by invasion with various spoilage organisms (Coxin et al., 1973). Two other phytoalexins— β -glucosides of 6-methoxymellein and 6-hydroxymellein—also are produced by carrot cells (Kurosaki et al., 1984). Production of 6-methoxymellein is elicited by fungal invasion (Amin et al., 1986a; Kurosaki and Nishi, 1983) and also by partial hydrolysis of carrot cells (Kurosaki and Nishi, 1984). The compound is toxic to bacteria as well as to yeasts and molds. Inhibition of gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*) and gram-negative bacteria (*Salmonella typhimurium*, *Shigella sonnei*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus vulgaris*) was reported when cells were exposed to 0.05 to 0.5 millimole methoxymellein (Kurusaki and Nishi, 1983). The mechanism of action of this phytoalexin

evidently involves interference with membrane associated functions. Alteration of membrane permeability (Amin et al., 1988; Weinstein and Albersheim, 1983), mitochondrial function (Boydston et al., 1983), cyclic nucleotide phosphodiesterase activity, and multilamella liposomes (Amin et al., 1986b) has been documented. The broad spectrum of antimicrobial activity exhibited by 6-methoxymellein and other elicited or naturally present compounds in carrots is particularly attractive relative to the potential use of these compounds as food preservatives.

Shredded carrots and carrot juice have a lethal effect on *Listeria monocytogenes*. Beuchat and Brackett (1990) reported that viability of the bacterium decreased upon contact with raw carrot but not with cooked carrot, indicating that the lethal compound(s) is(are) heat labile. In a study using *L. monocytogenes*, *L. innocua*, *L. ivanovi*, *L. seeligeri*, and *L. welshimeri*, Nguyen-the and Lund (1991) confirmed that a lethal component or components existed in raw sliced and macerated carrots. In a subsequent study (Nguyen-the and Lund, 1992), it was shown that the antilisterial activity of carrot slices was suppressed by anaerobiosis, thiol compounds, and bovine serum albumin, but not by sodium ascorbate, propyl gallate, catalase, superoxide dismutase, or chelating agents.

Extracts of carrot roots inhibit differentiation and aflatoxin formation by *Aspergillus parasiticus* (Batt et al., 1980). Geraniol, citrol, and terpineol in the volatile fraction of carrot seed oil prevent growth, whereas limonene and terpinene do not affect growth but do inhibit aflatoxin production (Batt et al., 1983). Whether these compounds also would influence growth patterns of microflora naturally present on carrots and other fresh produce is unknown.

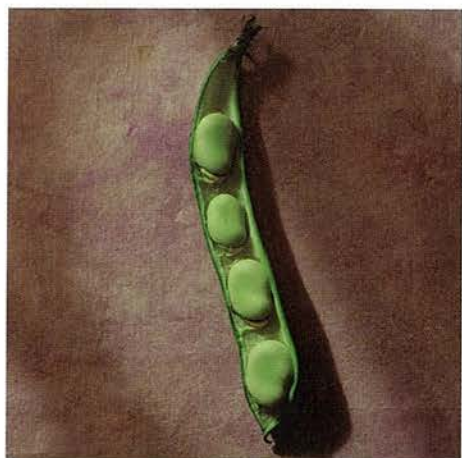


Figure 3.24. Pisatin is an antimicrobial phytoalexin present in peas.

Polypeptide Antimicrobials

Introduction

Among the natural substances considered safe alternatives to synthetic chemical preservatives in food processing are various enzymes and other peptides. Some polypeptides are being used while others seem promising for food preservation. Polypeptides with antimicrobial properties have been isolated from all plants and animals in which they have been sought (Gabay, 1994; Owen Fields, 1996). Polypeptides present in animal or plant tissue that are important in defense against pathogenic microorganisms often are classified as functioning by oxygen dependent or independent mechanisms. The oxygen dependent de-

Table 3.5. Phytoalexin production by edible plants (adapted from Whitehead and Threlfall, 1992)

Family genus	Common name	Elicitor	Phytoalexin formed	Reference
Composite				
<i>Carthamus tinctorius</i>	Sunflower	<i>Phytophthora megasperma</i> , <i>Alternaria carthami</i>	Safynol, dehydrosafynol, tridecentetrayne, epoxide derivative	Tietjen and Matern (1984)
Convolvaceae				
<i>Ipomoea batatas</i>	Sweet potato	Yeast extract	Dehydroipomeamarone, ipomeamarone, ipomeamaronol, 4-hydroxydehydromyoporone, 4-hydroxymyoporone	Oba and Uritani (1979)
Leguminosae				
<i>Arachis hypogaea</i>	Peanut	<i>Phytophthora cambivora</i>	Resveratrol, 4-isopentenyl resveratrol, 3,3,5-tri-hydroxy-4-methoxystilbene	Fritzemeier et al. (1983), Rolfs et al. (1987)
<i>Canavalia ensiformis</i>	Jack bean	<i>Pithomyces charatarum</i>	Medicarpin	Gustine et al. (1978)
<i>Cicer arletinum</i>	Chick pea	Yeast extract	Maackian	Kessman and Barz (1987)
<i>Glycine max</i>	Soybean	<i>Phytophthora</i> species, <i>Pseudomonas</i> species	Glyceollin I, II, III, daidzein, coumestrol	Keen and Horsch (1972) Ebel et al. (1976), Reilly and Klarman (1980), Fett and Zacharius (1983)
<i>Medicago sativa</i>	Alfalfa	<i>Fusarium oxysporum</i> , <i>Verticillium alboarum</i>	Medicarpin	Gustine and Moyer (1982), Latunde-Dada et al. (1987)
<i>Phaseolus vulgaris</i>	French and dwarf beans	<i>Aspergillus niger</i> , <i>Botrytis cinerea</i> , <i>Colletotrichum linemuthianum</i> , <i>Marcophonia phaseolina</i> , <i>Rhizopus arrhizus</i> , <i>R. solani</i>	Daidzein, genistein, 2-hydroxdaidzein, 2-hydroxygenistein, Kievitone, phaseollidin, phaseollinisoflavan, phaseollin	Dixon and Fuller (1976, 1978), Hargreaves and Selby (1978), Robbins et al. (1985), Peleg et al. (1987), Ellis et al. (1989)
<i>Pisum sativum</i>	Pea	Coconut milk	Pisatin	Bailey (1970)
<i>Vicia faba</i>	Broad bean	none	Wyerone, wyerol, wyeronic acid, dihydrowyerone	Wolff et al. (1988)
Malvaceae				
<i>Gossypium</i>	Cotton	<i>Saccharomyces cerevisiae</i> , <i>Verticillium dahliae</i>	Gossypol, hemigossypol, g-Q-methylgossypol, 6-Q-hemigossypol	Altman et al. (1985), Heinstein (1985)
Solanaceae				
<i>Capsicum annum</i>	Sweet pepper	<i>Trichoderma vivide</i> , <i>Glilocladium deliquescens</i>	Capsidiol	Brooks and Watson (1985)
<i>Lycopersicon esculentum</i>	Tomato	<i>Photophthora infestans</i>	Unidentified	Scala et al. (1983)
<i>Solanum melongena</i>	Eggplant	RNase	Falcarindiol, hydroxyfalcarinidiol, cis-penta-deca-6-ene-1,3-diyne-5, 15-diol	Imoto and Ohta (1988)
<i>Solanum tuberosum</i>	Potato	<i>Phytophthora infestans</i> , arachidonic acid	3-Hydroxylubimin, lubimin, phytuberin, rishitin, solaretivone	Ersek and Sziraki (1980), Brindle et al. (1983, 1988)
Umbelliferae				
<i>Petroselinum</i>	Parsley	<i>Alternaria carthamal</i> , <i>Physophthora megasperma</i> , chitosan	Begapten, graveolone, isopimpinellin, psoralen, xanthotoxin	Tietjen et al. (1983), Kombrink and Halbrook (1985), Conrath et al.(1989)
<i>Daucus carota</i>	Carrot	<i>Botrytis cinerea</i> , <i>Chaetomium globosum</i> , <i>Furarium moniliforme</i> , <i>Helminthosporum oryzae</i> , <i>Aspergillus niger</i>	6-Hydroxymellein, 6-methoxymellein	Kurosaki et al. (1984, 1985, 1986), Amin et al. (1986a,b), Bruemmer and White (1986), Marinelli et al. (1990)

1922). Lysozyme in the white (*albumen*) of chicken eggs (Figure 3.26) comprises up to 3.5% of protein content and can be extracted economically from this source. Lysozyme from hen egg white is a single polypeptide of 129 amino acids, with a molecular weight of approximately 14,700 Da, and is linked in four places with disulfide bridges (Tranter, 1994). At least two of these linkages must be intact for biological activity to be present. It is a basic protein and has an isoelectric point of 10.5 to 11. Lysozyme was the first enzyme whose structure was determined by x-ray diffraction; it appears as roughly ellipsoidal with dimensions of about 45 × 30 × 30 Å, with most of the

nonpolar chains facing inwards (Blake et al., 1965).

Lysozyme has desirable properties as a food preservative (Cunningham et al., 1991; Johnson, 1994). For instance, it is economical to produce from egg whites in good yield and purity by ion-exchange resins. It has very low or no toxicity when consumed orally at levels exceeding 0.2%, even after extended periods of consumption. It has a very specific target directed against bacterial and fungal cell walls (Jolles and Jolles, 1984), and because it is odorless with a slightly sweet taste, it does not interfere with the sensory qualities of products, particularly because the quantity used in foods is very low (0.002 to 0.04%).

Egg white lysozyme remains stable despite a number of food processing operations. It can withstand boiling for 1 to 2 min at acidic pH values in solution but is denatured upon boiling at high pH values. At pH 8.0, lysozyme was destroyed by heating at 65°C for 15 min, but no loss was recorded when heated at pH 5.0 for 60 min (Tranter, 1994). Heat inactivation generally is lower at pH values below 7.0. Lysozyme is not denatured by organic solvents such as ethanol used in foods. The enzyme can be frozen and is stable to spray drying. It is active from about 1°C to near boiling, and its optimal pH range is from 5.3 to 6.4. These properties contribute to its usefulness as an antimicrobial in low-acid refrigerated foods or in alcoholic beverages.

Antimicrobial activity against bacteria is limited by the masking of the peptidoglycan by outlying polymers and by substitution of peptidoglycan with chemical groups. Hen egg white lysozyme is active primarily against specific groups of gram-positive bacteria. For example, it is very active against the gram-positive organism *Micrococcus luteus* (*lysodekticus*) and against certain nonpathogenic *Clostridium*, *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Corynebacterium*, and *Streptococcus* spp. (Carini et al., 1985). Because of its specificity, egg white lysozyme generally does not interfere with food and beverage fermentations carried out by beneficial lactic acid bacteria and yeasts.

Hughey and Johnson (1987) found that egg white lysozyme, in addition to acting against spoilage bacteria, prevented growth of lysed bacterial foodborne pathogens, including *Listeria monocytogenes* and *Clostridium botulinum*. The activity of lysozyme against bacteria is enhanced by compounds acting in synergy and enabling lysozyme to penetrate to the peptidoglycan substrate. A particularly effective synergist for lysis of gram-negative as well as for gram-positive bacteria is EDTA (Repaske, 1958), which allows penetration of lysozyme to the peptidoglycan

Table 3.6. Biological properties of proteins of avian egg albumen (from Tranter, 1994)

Protein	Molecular mass (D ^a)	Biological properties
Ovalbumin	46,000	Phosphoglycoprotein
Ovotransferrin	78,000	Chelation of metal ions, especially Fe ³⁺
Ovomucoid	28,000	Inhibition of serine proteases
Lysozyme	14,600	Hydrolysis of β (1–4) glycosidic bonds of bacterial cell peptidoglycan; maintains integrity of albuminous sac by electrostatic interaction with ovomucin
Ovomucin	Not reported	See above
Ovoinhibitor	44,000–49,000	Inhibition of serine proteases
Ovoflavoprotein	32,000	Chelation of riboflavin
Avidin	66,000–69,000	Chelation of biotin
Ovoglycoprotein	24,400	—
Cystatin	13,143	Inhibition of cysteine proteases
Ovomacroglobulin	760,000–900,000	—

^a Daltons.



Figure 3.26. Hens' eggs contain lysozyme.

target.

In the presence of EDTA, gram-negative pathogens and spoilage bacteria, including *Salmonella*, *Campylobacter*, *Escherichia*, *Yersinia*, and *Pseudomonas* are lysed. Chelators compatible with foods, including polyphosphates, and certain organic acids and food preservatives also promote the activity of lysozyme, but generally to a lesser degree than EDTA does. Hen egg white lysozyme was stable *in vitro* in combination with other antimicrobial substances, including 1% sodium chloride, 0.01% sodium nitrite, 4% ethanol, 0.01% hydroxytoluene, 0.1% sodium benzoate, 0.3% calcium propionate, 0.1% potassium sorbate, and 0.1% propyl paraben; activity was lost, however, in combination with 0.5% EDTA, 0.5% lactic acid, 4% acetic acid, and 0.01% chlorine compounds in water (Yang and Cunningham, 1993).

Physical treatments such as sublethal heating or exposure to low pH also can enhance lysozyme activity. The activity of lysozyme against *Listeria monocytogenes* was highest at low temperatures (Smith et al., 1991), a fact indicating that the enzyme may be particularly useful in refrigerated foods. Lysozyme also may bind or be sequestered by food components, which can decrease its activity; it may be most useful in fairly homogeneous food systems such as juices and alcoholic beverages.

Pathogens inhibited by lysozyme in foods include *Salmonella senftenberg* in poultry (Samuelson et al., 1985; Teotia and Miller, 1975); *Listeria monocytogenes* also was inhibited by egg white lysozyme in culture media and in foods (Hughey and Johnson, 1987; Hughey et al., 1989). Growth inhibition by lysozyme was promoted, not only by EDTA, but also by lactic acid, trypsin, proteinase K, conalbumin, lactoferrin, and lipase; removal of certain proteins therefore influences the ability of lysozyme to penetrate and to hydrolyze the peptidoglycan of the microbial cell wall (El-Kest and Marth, 1992).

Lysozyme inactivated or prevented growth of *Listeria monocytogenes* Scott A in several vegetables but was less effective in animal derived foods (Hughey et al., 1989). *Listeria monocytogenes* survived extremely well in whole milk and also was very resistant to lysozyme (Carminati and Carini, 1989; Hughey et al., 1989). Growth of *L. monocytogenes* at 4°C was decreased by lysozyme, however. Several trials were conducted in soft-ripened cheeses to control *L. monocytogenes* by means of lysozyme (Hughey et al., 1989), but the pathogen was able to grow well. Growth of *L. monocytogenes* probably is associated with the rise in pH occurring during ripening of soft cheeses. In general, *L. monocytogenes* survives poorly in egg albu-

men. Erickson and Jenkins (1992) found that *L. monocytogenes* was inactivated in unsalted pasteurized egg white and whole egg. Wang and Shelef (1991) concluded that the antilisterial activity of egg albumen was due primarily to lysozyme and was enhanced by ovomucoid, conalbumin, and alkaline pH. Their data support the assumption that lysozyme is listericidal and suggest that other unidentified components in egg albumen may enhance its activity.

The public health safety of low-acid foods depends on the control of *Clostridium botulinum* to prevent botulin toxin formation, and many food preservation procedures in current use are designed to control this important pathogen. Certain strains of "group II" *C. botulinum* (Smith and Sugiyama, 1988), composing serotypes B, E, and F, could proliferate in these foods and present a botulism hazard (Conner et al., 1989). Commercial developments also have promoted the use of fish proteins in meat and poultry products, which could increase the possibility of a hazard from *C. botulinum* where it did not exist before. The growth potential of *C. botulinum* also is higher in products developed recently, such as vacuum-packaged and modified atmosphere foods (Farber, 1991), than in traditional food products. The food industry would benefit from the development of improved safety systems to control *Clostridium botulinum* in low-acid refrigerated foods. Egg white lysozyme is known to inactivate or to prevent growth of spores or vegetative cells of *C. botulinum* in culture media and in several foods, including turkey meat, fresh pork sausage, salmon, asparagus, potatoes, tomatoes, and mushrooms. As with other organisms, antibotulinal activity is enhanced in the presence of chelators or other synergistic agents.

New formulations and derivatives of lysozyme could further improve its effectiveness in foods. Using the Maillard reaction, Nakamura et al. (1991) prepared lysozyme-dextran conjugates, which had improved emulsifying properties and inhibited both gram-negative (*Vibrio parahaemolyticus*, *Escherichia coli*, *Aeromonas hydrophila*, and *Klebsiella pneumoniae*) and gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). The emulsifying properties of the conjugate evidently allowed penetration of lysozyme through the outer membrane layers to the peptidoglycan substrate. A derivative of lysozyme prepared by means of the N-hydroxysuccinimide derivative of palmitic acid was more active against *E. coli* but less active against *Micrococcus lysodeketicus* than lysozyme was (Ibrahim et al., 1991). This work indicated that a novel class of antimicrobials with increased specificity could be developed by modification

of lysozyme to allow penetration through outer membranes to the peptidoglycan substrate.

In addition to its potential use as a food preservative, lysozyme long has been thought to function as a probiotic or "humanization" agent in milk and infant formulae. When administered in baby formula, it encourages the growth of *Lactobacillus* spp. in the intestine of human infants, thereby discouraging the growth of undesirable organisms (Schwimmer, 1981). Lysozyme also has been reported to influence behavior and immunological resistance. The release of muramyl peptides contributes to sleep, immunopotentialization, and nutrition (Adam and Lederer, 1984; Takada and Kotani, 1985).

An interesting new development is the expression of lysozyme activity in heterologous hosts. The genes for human, bovine, and microbial lysozymes have been cloned and in some instances expressed in microorganisms, some of which are compatible with foods. The gene for hen egg white lysosome was expressed in *Lactococcus lactis* as determined by immunological analysis, but no lysozyme activity was observed, presumably because disulfide bonds were formed incorrectly (Van de Guchte et al., 1992). Hen egg white lysozyme has been expressed in an active form in food-compatible microorganisms including *Saccharomyces cerevisiae* and *Aspergillus niger* (Archer et al., 1990; Oberto and Davison, 1985). Heterologous expression of active lysozyme in transgenic plants has been proposed as a means of preventing disease and plant spoilage.

Other Lytic Enzymes

As with lysozyme, enzymatic degradation of the cell surface of undesired organisms can be used to control microbial growth in foods. In addition to lysozyme, a number of other enzymes are known that specifically cleave carbohydrate or peptide linkages in the cell wall of bacteria and fungi. This complement of enzymes includes glycosidases specific for the glycoside bond between alternating sugars in the polysaccharide backbone, an amidase releasing the tetrapeptide from N-acetylmuramic acid, endopeptidases attacking bonds in the peptide bridge, and lipases hydrolyzing attached lipids and membranes. These enzymes could be useful in the control of bacteria in foods even though they have not served useful purposes as therapeutic agents in humans, because of interfering immune reactions (Leive and Davis, 1980). Many of the lytic enzymes have a specificity for bacterial species that is markedly different from that of hen egg white lysozyme. These enzymes have potential value in food preservation or as animal feed adjuncts but most have

not been tested in foods.

Three bacteriolytic enzyme mixtures were examined for lysis of various bacteria and were compared to lysozyme (Nielsen, 1991). An enzyme mixture (SP416) produced by *Nocardiosis dassonvillei* was reported to lyse *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The enzyme seemed to act synergistically with the protease subtilisin, was active over the broad pH range of 5 to 9, and had an optimal temperature of 50°C. Two N-acetyl-hexosaminidases were purified from the bacteriolytic enzyme mixture, suggesting it had a mechanism similar to lysozyme. A bacteriolytic enzyme mixture (SP417) produced by *Bacillus pabuli* was reported to lyse gram-negative bacteria preferentially, including *Escherichia coli*, *Salmonella arizonae*, *P. aeruginosa*, and *Vibrio parahaemolyticus*. The enzyme was active from pH 5 to 7 and at temperatures as high as 60°C. A third enzyme mixture produced by a strain of *N. dassonvillei* was most active against *P. aeruginosa*. The preferential activity of certain of these mixtures on gram-negative bacteria is intriguing and suggests that they are able to penetrate the outer membrane or that their activity is potentiated by other factors present in the mixtures. The activity of the enzymes was strain-dependent, however, and not all isolates of target organisms were lysed. Nielsen (1991) reported that the lytic enzymes lysed bacterial pathogens, including *Campylobacter fetus*, *E. coli*, *Salmonella* serovars, and *V. parahaemolyticus*, a fact indicating that they may be useful as natural food antimicrobials. It was suggested that the bacteriolytic enzymes could be used to extend the shelf-life of unprocessed or minimally processed foods.

Streptomyces spp. secrete several enzymes with cell-wall degrading activity that could have applications in food preservation. *Streptomyces coelicolor* secretes a lysozyme called *cellosyl* with both β -1,4-N-acetyl- and β -1,4-N,6-diacetyl muramidase activities (Brau et al., 1991). The enzyme degrades the cell wall of *Staphylococcus aureus*, which is resistant to degradation by egg white or phage lysozymes. An enzyme with N-acetylmuramidase activity from *Streptomyces rutgersensis* was suggested as a food preservative for bean paste (*an*) made of adzuki beans (Hayashi et al., 1989). The shelf-life of the product was extended from 48 to 109 hours at 10°C at a rather high enzyme concentration of 0.11% (hen egg lysozyme generally is used at approximately 0.0005 to 0.01%). The shelf-life of raw *an* was increased from 48 to 109 hours with 0.109% lytic enzyme at 10°C. Certain compounds acted synergistically with lysozyme, increasing the shelf-life to 138 hours (acetic acid, 0.1%), 123 hours (lactic

acid, 0.15%), 120 hours (glycine, 1%), or 117 hours (potassium sorbate, 0.02%); whereas EDTA did not prolong shelf-life, and ethyl alcohol shortened it to 68 hours.

Lytic enzymes differ in their modes of action and in their specificities for the cell walls of target organisms. These properties suggest that combinations of enzymes would be optimal for certain applications. Roberts and Selitrennikoff (1988) showed that plant and bacterial chitinases had widely different antifungal activities. Chitinases were isolated from the grains of wheat, barley, and maize and were compared with chitinases from *Streptomyces griseus* and *Pseudomonas stutzeri* for antifungal activity. Antifungal activity was detected in 1 g of the grain chitinases whereas no bacterial chitinase was active. This difference in activity was explained in terms of the different mechanisms of action of the chitinases. The grain chitinases, like the other plant chitinases, functioned as endochitinases and contained lysozyme activity. These experiments supported the hypothesis that plant chitinases could be used to protect against fungal colonization.

Mutanolysin, a muramidase produced by *Streptomyces globisporus*, has been used to digest cell envelopes from a number of bacterial species (Yokogawa et al., 1975). Mutanolysin was active on cariogenic bacteria, including *Streptococcus mutans*, and when injected into the inflamed joint also may alter the course and the severity of erosive arthritis in mammals (Janusz et al., 1984). Its potential use in foods does not seem to have been investigated.

A lysozyme isolated from rainbow trout was reported to have lytic activity on mastitis pathogens (staphylococci, streptococci, and coliforms) (Grinde, 1989). The enzyme was effective against *Staphylococcus*, whereas egg lysozyme and mutanolysin had little effect. Lysostaphin also was lytic, and it is well known to have lytic activity against *Staphylococcus* spp. The purpose of the work (Grinde, 1989) was to investigate antimicrobial proteins that could be used in a transgenic strategy to increase disease resistance. This strategy also could be used to decrease spoilage of raw foods. In summary, research with the lytic enzymes of microbial origin suggests that individual enzymes, e.g., lysozyme, or mixtures of enzymes with different activities and specificities could be used as nontoxic food preservatives.

Peroxidases and Oxidases

Introduction

The presence or absence of oxygen contributes greatly to the microbiological status of foods. Certain microorganisms are inhibited by deprivation of oxygen, and others through inactivation caused by toxic oxygen metabolites, including hydrogen peroxide, superoxide ion, singlet oxygen, and the hydroxyl radical. *Oxidases* are enzymes oxidizing organic substrates, with molecular oxygen generating hydrogen peroxide. Oxidases can inhibit microorganisms by producing hydrogen peroxide and, in the presence of catalase, by depriving an organism of oxygen. Xanthine oxidase is an example of a naturally occurring enzyme in milk that generates hydrogen peroxide.

Peroxidases are widespread in nature and oxidize molecules at the expense of hydrogen peroxide (Reiter and Harnulv, 1984), according to the reaction $H_2O_2 + X^- \rightarrow H_2O + OX^-$. There are many peroxidases with different redox potentials and substrate specificities (Ekstrand, 1994). Lactoperoxidase oxidizes thiocyanate or halogens, thereby producing toxic metabolites. Often, the rate-limiting substrate for peroxidase activity in foods is the availability of hydrogen peroxide, which is reactive and must be provided continuously. Hydrogen peroxide can be provided through the use of lactic acid bacteria, which generate hydrogen peroxide through their metabolism, or through the use of glucose oxidase, which produces it from oxygen and glucose, or from chemical reagents such as sodium bicarbonate.

Lactoperoxidase and Related Peroxidases

Peroxidase activity first was discovered in cow's milk at the end of the nineteenth century (Ekstrand, 1994). Lactoperoxidase, a product of the mammary glands and the most abundant enzyme in bovine milk, also is produced in salivary glands and is present in the saliva of mammals. Lactoperoxidase has been well documented to be involved in the natural antibacterial activity present in cow's milk and actually may be involved sometimes in inhibiting starter cultures used in cheese production (Reiter and Harnulv, 1984). The enzyme, together with lactoferrin, can be isolated from sweet whey concentrate through a simple procedure using cation-exchange resin. The procedure is amenable to scaling up for production in industry (Yoshida and Xiuyun, 1991). Lactoperoxidase is present in milk at 0.002 to 0.003%, where it accounts for 2 to 3% of total whey protein and is found in milk from many species and in many types of secretions, e.g., tears, saliva, and nasal fluid (Ekstrand, 1994).

Bovine milk lactoperoxidase, reasonably heat resistant and inactivated only partly by pasteurization at 74°C (Reiter and Harnulv, 1984), might survive the minimal heat processing of certain foods.

Lactoperoxidase, a basic heme-containing protein with a high isoelectric point, is comprised of a single polypeptide, of which approximately 10% is carbohydrate. The amino acid sequence of bovine lactoperoxidase has been determined, and it is similar in structure to human myeloperoxidase, eosinophil peroxidase, and thyroid peroxidase (Cals et al., 1991; Dull et al., 1990). The single peptide chain of bovine lactoperoxidase is comprised of 612 amino acid residues with 15 half-cystines and 4 to 5 potential *N*-glycosylation sites (Ekstrand, 1994). Molecular mass is estimated at 78.5 kDa. Lactoperoxidase is prepared from bovine milk whey by means of ion exchange chromatography on a cation exchanger (Yoshida and Xiyun, 1991).

Lactoperoxidase binds hydrogen peroxide and catalyzes the oxidation of electron donors such as the thiocyanate ion (i.e., SCN⁻) or the iodide ion (I⁻) to intermediate oxidation products (e.g., hypothiocyanate; OSCN⁻) with antimicrobial activity. Thus, the antimicrobial activity of the lactoperoxidase system depends on the availability of all three components. The amounts of the three components, i.e., lactoperoxidase enzyme, thiocyanate, and hydrogen peroxide, required for antibacterial activity are low, i.e., 0.0012 and 0.008%, respectively, for SCN⁻ and hydrogen peroxide (International Dairy Federation, 1988). In milk, lactoperoxidase oxidizes thiocyanate with hydrogen peroxide to intermediate oxidation products, including hypothiocyanate ions, i.e., OSCN⁻ and O₂SCN⁻ (Pruitt and Reiter, 1987). This product and other, more unstable, oxidation products react directly with proteins of microbial cell membranes and thus interfere with metabolism and cell division.

The antimicrobial activity of the lactoperoxidase system against microorganisms is both species and strain specific. The system is bactericidal to gram-negative bacteria but usually only bacteriostatic or temporally inhibitory to gram-positive organisms. In raw milk, the lactoperoxidase system inhibits fermentation starter organisms such as lactic acid producing bacteria but is bactericidal to gram-negative spoilage psychrotrophs and to *Salmonella* (Reiter and Harnulv, 1984). Growth phase, stress, and cell envelope structure affect the susceptibility of *S. typhimurium* to the lactoperoxidase system (Leyer and Johnson, 1993). Actively growing cells are more easily inactivated than salmonellae in the stationary phase of growth (Purdy et al., 1983). Hence, the food

composition and the physiological state of the target microorganisms would affect susceptibility of undesirable microorganisms to lactoperoxidase. *Campylobacter* strains isolated from poultry were inactivated by the lactoperoxidase system in ultra high-temperature (UHT) milk (Borch et al., 1989).

The lactoperoxidase system is thought to be less active against gram-positive pathogens, and it inhibited but did not inactivate *Bacillus cereus* (Zajac et al., 1981) and *Streptococcus uberis* associated with mastitis infections (Marshall et al., 1986). The lactoperoxidase system also was bacteriostatic but did not prevent eventual growth of *Listeria monocytogenes* in skim and whole milk and in cottage cheese (Earnshaw and Banks, 1989; Earnshaw et al., 1989; Kamau et al., 1990a; Siragusa and Johnson, 1989). The lactoperoxidase system did, however, eliminate *L. monocytogenes* from French soft cheese (Denis and Ramet, 1989). The bacteriostatic or bactericidal activity of the lactoperoxidase system against *L. monocytogenes* depended on temperature, incubation time, and *L. monocytogenes* strain (Gaya et al., 1991). The lactoperoxidase system sensitized most *L. monocytogenes* and *Staphylococcus aureus* cells to heat inactivation, but a subpopulation of each organism seemed resistant (Kamau et al., 1990b).

The lactoperoxidase system can be inactivated by sulfhydryl (SH) groups that either bind directly to the heme group or scavenge OSCN⁻ and thus compete with the target substance. Presence of lactoperoxidase in fresh milk is sufficient for the system to operate whereas SCN concentration depends on the feed, and hydrogen peroxide must be added, generally chemically, or produced by lactic acid bacteria (Ekstrand, 1994).

Toxicological hazards associated with use of the lactoperoxidase system to preserve milk are minimal (Farrag and Marth, 1992); generally, the system is harmless to the host organism (Ekstrand, 1994). The toxicological safety of the lactoperoxidase system is supported by the detection of some lactoperoxidase system oxidation products in human saliva and the selective inactivation of bacterial, although not mammalian, membranes by oxidation products (Reiter and Harnulv, 1984). Mammals also contain inherent related peroxidases, including neutrophil myeloperoxidase, eosinophil peroxidase, thyroid peroxidase, intestinal mucosal peroxidase, and uterine peroxidase, all of which are microbicidal in human tissue.

Other Peroxidases

In addition to lactoperoxidase, several microbicidal peroxidases are present in mammalian tissues and

secretions. Neutrophil granules contain various polypeptides, including cationic peptides, lactoferrin, and the unusual enzyme myeloperoxidase, with bactericidal properties (Hurst and Barrette, 1989). The myeloperoxidase hydrogen peroxide chlorine system comprises about 1 to 5% of the dry weight of protein in these mammalian cells. Myeloperoxidase binds hydrogen peroxide, which oxidizes Cl^- to hypochlorous acid (HOCl). The HOCl is quite reactive and bactericidal in the low pH of the phagosome (Albrich et al., 1981; Rosen and Klebanoff, 1982). The system also may halogenate microbial components to form N-chloramines, which are highly microbicidal. Hypochlorous acid preferentially attacks electron-rich biological targets, including nitrogen bases of amino acids and amines, sulfhydryl groups, and iron-sulfur clusters.

Such powerful bactericidal enzymes might be useful in specific foods that do not have a high ratio of quenching targets to undesirable organisms (Banks et al., 1986), but the system would act similarly to lactoperoxidase in that it would have little or no selectivity and could generate toxic by-products. Hence, system utility would be greatest in raw foods, from which elimination of the entire microbiological flora is desired.

Glucose Oxidase and Other Oxidases

Glucose oxidase catalyzes a reaction between glucose and oxygen and yields gluconic acid or D-glucono- δ -lactone and hydrogen peroxide. This colorless and tasteless enzyme has been approved to remove glucose from food and as an antioxidant. The enzyme also is known as glucose-aerohydrogenase, aero-glucose-hydrogenase, glucose oxyhydrogenase, or notatin and is used in the food industry to remove glucose from foods such as eggs, to prevent Maillard browning reactions, and to remove oxygen from beverages (Frank, 1992).

Little research has examined the effectiveness of glucose oxidase-glucose against foodborne pathogens. Tiina and Sandholm (1989) reported significant growth inhibition by glucose oxidase (0.5 to 1.0 international units [IU] per mL)-glucose (0.05 to 0.1%) of the pathogens *Salmonella infantis*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus* in sterile filtered meat medium. Other pathogens—*Campylobacter jejuni*, *Listeria monocytogenes*, and *Yersinia enterocolitica*—and the lactic acid bacterium *Lactobacillus plantarum* were relatively resistant in sterile meat medium but were more sensitive in heat denatured meat medium. The enzyme system also was reported to be effective in vitro in inhibiting the

growth of mastitis pathogens (Sandholm et al., 1988). Bacteria usually found on shrimp, including *Pseudomonas fluorescens*, *Acinetobacter calcoaceticum*, and *Corynebacterium aquaticum*, and the yeast *Hansenula polymorpha* also were inhibited by the system (Kantt and Torres, 1993). Inhibition of the spoilage organisms was due primarily to the production of hydrogen peroxide whereas *C. aquaticum* was inhibited by gluconic acid but unaffected by hydrogen peroxide.

Glucose oxidase did not decrease populations of *Salmonella* or *Pseudomonas* on chicken parts dipped in solutions containing the enzyme (Jeong et al., 1992). The preservative activity of glucose oxidase in fish and the lack thereof in poultry could be explained in terms of either the destruction of hydrogen peroxide by catalase or the microbial utilization of glucose (Jeong et al., 1992).

Several additional oxidase systems are known to be antimicrobial; these include xanthine oxidase and D-amino oxidase, which have broad antimicrobial activity against a range of organisms. The bactericidal activity of these systems seems correlated with the production of hydrogen peroxide through enzymatic catalytic activity (Skarnes, 1970). The potential uses of these oxidases, however, require evaluation in food systems.

Transferrins

Introduction

Growth and survival of many bacterial and fungal pathogens depend on the availability of iron ions, but absolute iron ion requirements have yet to be determined for most pathogens. The growth of bacteria of the genera *Staphylococcus*, *Clostridium*, *Listeria*, *Mycobacterium*, *Salmonella*, *Pseudomonas*, *Yersinia*, *Vibrio*, and *Aeromonas* is stimulated by iron ions (Weinberg, 1978). *Escherichia coli* requires 0.02 to 0.33 μg of iron per mL; *Pseudomonas*, which has a higher heme content, requires considerably more (Waring and Werkman, 1942). *Listeria monocytogenes* has a strict requirement for iron and an optimal concentration for growth of approximately 15 μg per mL (Premaratne et al., 1991). In contrast, lactic acid bacteria have a metabolism based on manganese ions instead of iron and have low iron requirements of 0.0002 to 0.01 μg per mL, depending on the strain (Reiter and Oram, 1968). It therefore is possible, by means of iron sequestration, to favor the growth of beneficial lactic acid bacteria over that of either pathogens or spoilage organisms in foods (Bruyneal et al., 1990). This can be accomplished through chelation by

iron-binding polypeptides, especially the transferrins and related proteins.

Ferrous and ferric ions are bound in tissues by certain proteins of the transferrin class. In serum and other body fluids, transferrins bind iron ions so tightly that the amount of free iron ions is about 100-millionfold less than that required for bacterial growth (0.4 to 4 μM). The iron-binding glycoproteins ovotransferrin and lactoferrin exert antimicrobial activity in biological fluids through their ability to withhold iron ions from microorganisms (Ekstrand, 1994). Another transferrin, serotransferrin, which occurs in blood plasma, transports iron ions.

Ovotransferrin or Conalbumin

The composition of egg white includes about 13% of its protein as the iron binding compound ovotransferrin, i.e., conalbumin. In 1944, raw egg white supplemented into nutrient broth was found to inhibit growth of *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, and *Saccharomyces cerevisiae* (Schade and Caroline, 1944). Inhibition was observed at a pH of 7.4 or above, but not at 5.8, and inhibition was reversed by iron. The iron-binding polypeptide was isolated and identified as conalbumin, which, as indicated, belongs to the class of proteins known as transferrins. Unlike antimicrobial enzymes such as lysozyme and lactoperoxidase, which act catalytically, conalbumin or other transferrins must be present in stoichiometric excess of the quantity of iron ions available. These proteins therefore would be useful only in foods such as milk or egg albumen, which have low iron content.

Ovotransferrin may be isolated from hen egg whites by ethanol or ammonium sulfate precipitation/fractionation or by ion exchange chromatography and metal affinity chromatography (Tranter, 1994). Ovotransferrin consists of a single polypeptide chain of more than 700 amino acid residues. In addition to iron (III)-binding ovotransferrin, other transferrins bind a variety of di- and trivalent metal ions. The complexes formed, i.e., metal-proteins, dissociate in a solution of pH < 6.5; thus, binding is more effective in alkaline environments. Metal binding is activated by oxalate, citrate, or EDTA. Conalbumin is denatured easily by heat, e.g., greater than 80% is denatured in 5 min at 60°C (Tranter, 1994).

The antimicrobial activity of conalbumin and other transferrins is based on the essential nature of iron ions for virtually all bacteria, either as a prosthetic group or as a cofactor in many enzymatic reactions. In living systems, transferrins transport iron ions to specific membrane receptors and ensure that micro-

organisms have available only limited iron ions to infect the biological fluids containing proteins (Tranter, 1994). Ovotransferrin is believed to be the key factor responsible for microbial inhibition in egg albumen, for its activity is enhanced in albumen's alkaline pH (8.5 to 9.5) whereas microbial growth resumes when the albumen is saturated with iron ions. To function at times of iron ion depletion, bacteria possess mechanisms such as the siderophores, which compete for low levels of iron ions and then form complexes with iron-repressible outer membrane proteins to supply cells with the iron needed for growth. Other bacteria can use transferrin iron directly and without the mediation of siderophores (Tranter, 1994).

Lactoferrin and Lactoferricin

Lactoferrin, present in milk (Figure 3.27), is another member of the transferrin family of iron-binding glycoproteins (Groves, 1960) and has been detected in tears, saliva, and mucosal secretions and in the granules of polymorphonuclear leukocytes (Masson et al., 1966, 1969). Lactoferrin is present in human colostrum at levels as high as 0.6% but decreases to approximately 0.1% in mature milk (Ward et al., 1992). Lactoferrin is present at much lower levels (0.002 to 0.02%) in bovine colostrum and milk (Reiter, 1978).

Lactoferrin, a 78-kDa glycoprotein binding two molecules of ferric iron, was purified and characterized in the 1960s as a major protein of human milk. Methods used to separate lactoferrin include various types of chromatography including metal chelate interaction chromatography. Purified lactoferrin is composed of two protein bands with an isoelectric point of 9.5. The gene of bovine lactoferrin has been cloned. Bovine lactoferrin is 69% identical to human lactof-



Figure 3.27. Lactoferrin, a protein present in milk, exhibits antimicrobial activity.

errin (Ekstrand, 1994).

The physiological functions of lactoferrin may include (1) protection of young animals from enteropathogenic bacteria in the intestine, (2) protection of the nonlactating mammary gland against mastitis, (3) protection of young animals against free metal ions, (4) physiological transport and supply of iron ions to the young animal, and (5) immunoregulation (Ekstrand, 1994). Thus, lactoferrin may be at least partly responsible for the beneficial effects of breast-feeding and has many different regulatory roles in the immune system.

Arnold et al. (1977) showed that *Streptococcus mutans* and *Vibrio cholerae*, but not *Escherichia coli*, were killed by incubation with purified apolactoferrin and that bactericidal effect was contingent on the metal chelating properties of the lactoferrin molecule. A more extensive study (Arnold et al., 1980b) demonstrated that several organisms were sensitive to lactoferrin, including *S. mutans*, *E. coli* (nonenteropathogenic), *V. cholerae*, *Pseudomonas aeruginosa*, and the yeast *Candida albicans*. Resistant organisms included *Streptococcus pyogenes*, *Lactobacillus casei*, *Staphylococcus aureus*, *E. coli* O111, *Salmonella newport*, and *Shigella sonnei*. Payne et al. (1990) demonstrated that bovine apolactoferrin had bacteriostatic activity against four strains of *Listeria monocytogenes* and an *E. coli* at concentrations of 15 to 30 mg/mL in UHT milk. At 2.5 mg/mL the compound had no activity against *Salmonella typhimurium*, *P. fluorescens* and limited activity against *E. coli* O157:H7 or *L. monocytogenes* VPHI (Payne et al., 1994). Both lactoferrin susceptible and resistant organisms encompass a variety of types, including gram-positive and gram-negative bacteria, rods and cocci, and aerobes and anaerobes. Susceptibility depends on similarities in cell surface structure or to the mode of lactoferrin for each organism.

As has been indicated, lactoferrin from bovine or human colostrum is bacteriostatic by virtue of its ability to sequester iron ions, but it also is bactericidal by means of a mechanism not dependent on iron-binding alone (Arnold et al., 1977, 1980). In addition to metal chelation, lactoferrin and pepsin derived lactoferrin peptides damage the outer membrane of gram-negative bacteria (Yamaguchi et al., 1993). Lactoferrin can inactivate gram-negative bacteria alone or by sensitizing them to lysozyme (Ellison and Giehl, 1991; Suzuki et al., 1989b), with which lactoferrin forms a complex (Leffell and Spitznagel, 1972). Binding of human lactoferrin or EDTA to the outer membranes sensitizes *Escherichia coli* to inactivation by colicin (Ellison et al., 1988), which emphasizes the impor-

tance of the outer membrane in protecting gram-negative cells from inactivation by polypeptides. The target protein in the outer membrane of lactoferrin probably is a porin, which can be shielded by LPS in the membrane (Naidu et al., 1993).

Active peptides are derived from the molecule's N-terminal region distinct from iron-binding sites (Bellamy et al., 1992). The active peptides of human lactoferrin and bovine lactoferrin have been isolated by means of reverse-phase, high-performance liquid chromatography and have been sequenced. The antimicrobial peptide sequence consists of 25 amino acids, mainly of a loop of 18 amino acids connected by a disulfide bond. The sequence contains five arginine and three lysine residues, making it strongly cationic and suggesting that its mode of action is similar to that of the defensins. The bactericidal domain in lactoferrin helps explain the observations made earlier that lactoferrin had rapid lethal activity unaffected by iron, as well as bacteriostatic activity counteracted by that element. Considerable homology exists in the transferrin family. Human lactoferrin has 59 and 40% homology with human transferrin and ovotransferrin, respectively (Metz-Boutique et al., 1984). Active peptides from other transferrins and from food grade proteins likely can be isolated with antimicrobial activity.

Digestion of bovine lactoferrin with gastric pepsin yields a hydrolysate with antibacterial activity greater than that of native lactoferrin (Hoek et al., 1997; Tomita et al., 1992). The peptide, lactoferricin B, inactivated a broad range of gram-positive and gram-negative bacteria, including *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Streptococcus mutans*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, and *Clostridium perfringens*. Resistant organisms included *P. fluorescens*, *Enterococcus faecalis*, and *Bifidobacterium bifidum*. Lactoferricin, effective at 0.00003 to 0.015%, depending on the microorganism, was active in the pH range of 5.5 to 7.5; it was most active under slightly alkaline conditions. Lactoferricin was lethal to four strains of *L. monocytogenes* at concentrations from 0.00003 to 0.0009% (Wakabayashi et al., 1992).

Other Metal-Binding Polypeptides

Metal chelation mechanisms seem to have considerable potential as natural antimicrobials for foods. Vertebrates and fungi possess, in addition to transferrins, metal-binding polypeptides that detoxify heavy metals. Metallothioneins are sulfur-rich por-

teins of about 6.5 kDa and are comprised of 61 amino acids in mammals. Novel heavy-metal complexing peptide phytochelatins are synthesized by higher plants (Grill et al., 1985). The structure of the phytochelatins was established as (gamma-glutamic acid-cysteine) n-glycine (n = 3 to 7). These relatively small peptides could be used to sequester metals in food preservation.

Antimicrobial Peptides

Introduction

Several classes of naturally occurring basic small molecular weight peptides have potent antimicrobial activity and could be used to control undesirable microorganisms in foods. The most prominent group of peptides are the defensins, but others such as lactoferricin, which is derived from hydrolysis of food-grade proteins such as lactoferrin, also have been isolated. Many of these antibacterial peptides occur naturally in mammals, including humans, and in insects in which they are involved in immunological protection against microbial invasion. The bactericidal cationic peptides also are related to bacteriocins produced by bacteria and to the cationic compounds protamine sulfate and aprotinin. Compared with certain enzymes used in foods, e.g., lysozyme and lactoperoxidase, many antibacterial peptides from human and animal sources are toxic to a certain degree towards their own cells, and their presence is limited to specific cell types (Casteels, 1990). The application of such enzymes to foods could be limited unless nontoxic derivatives are isolated. The widespread distribution of cationic peptides suggests that they have evolved as important groups of compounds for antibiosis.

Bacteriocins

Bacteriocins, or naturally produced, small peptides with bactericidal activity usually against closely related bacteria (Holz and Stahl, 1995; Hoover and Steenson, 1993) seem to have potential as natural antimicrobials for foods (McMullen and Stiles, 1996). A classic group of bacteriocins, discovered in 1925, is the colicins, which are released by certain strains of *Escherichia coli* and inhibit growth of a limited number of other strains (Hoover, 1992). A great number of bacteriocins subsequently were found in microorganisms such as *Bacillus*, *Bacteroides*, *Brucella*, *Carnobacterium*, *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Listeria*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, and many other genera of bacteria, including food fermentations bacteria (Table 3.7) such as *Lactococcus*, *Leuconostoc*, *Pediococcus*,

Lactobacillus, *Bifidobacterium*, and *Propionibacterium* (Barefoot et al., 1992; Hansen, 1994; Hoover, 1992; Jack et al., 1995; Muriana, 1996; Nettles and Barefoot, 1993; Ray and Daeschel, 1992; Tagg et al., 1976).

Antibacterial peptides often are produced by gram-positive starter culture bacteria associated with dairy, vegetable, and meat fermentations and by vegetative, gram-negative, enteric, and nonenteric bacteria. These peptides all are called *bacteriocins*, although their amino acid sequences, chemical properties, molecular weights, activity modes, and host specificities may differ substantially (Owen Fields, 1996). Basic and practical aspects of bacteriocins have been reviewed thoroughly (Klaenhammer, 1988; Marugg, 1991; McMullen and Stiles, 1996; Muriana, 1996; Owen Fields, 1996; Ray and Daeschel, 1992).

Bacteriocins are of two types—those with a narrow range of bactericidal activities against closely related species, and those with a broader range of activities against a wider range of microorganisms (Klaenhammer, 1993). Degree of specificity could dictate the type of foods in which an antimicrobial could be used. Molecules with a narrow specificity range could be targeted to inhibit individual species in a complex mixture of microorganisms as they occur in many com-

Table 3.7. Old and new names of lactic acid bacteria (from Dillon and Cook, 1994)

Old names	New names
<i>Lactobacillus acidophilus</i>	<i>Lactobacillus acidophilus</i>
<i>Lactobacillus bulgaricus</i>	<i>Lactobacillus delbrueckii</i> subsp. ^a <i>bulgaricus</i>
<i>Lactobacillus casei</i>	<i>Lactobacillus casei</i> subsp. <i>casei</i>
<i>Lactobacillus lactis</i>	<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>
<i>Lactobacillus oenos</i>	<i>Leuconostoc oenos</i>
<i>Leuconostoc citrovorum</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>
<i>Leuconostoc cremoris</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>
<i>Leuconostoc mesenteroides</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
<i>Pediococcus cerevisiae</i>	<i>Pediococcus acidilactici</i>
<i>Propionibacterium shermanii</i>	<i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i>
<i>Streptococcus citrovorus</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>
<i>Streptococcus cremoris</i>	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>
<i>Streptococcus diacetylactis</i>	<i>Lactococcus lactis</i> subsp. <i>lactis</i> serovar <i>diacetylactis</i>
<i>Streptococcus faecium</i>	<i>Enterococcus faecium</i>
<i>Streptococcus lactis</i>	<i>Lactococcus lactis</i> subsp. <i>lactis</i>

^aSubspecies

plex food fermentations. Broader-range bacteriocins could be used in raw foods, from which elimination of the entire microbial flora is desired.

Bacteriocins are not included in the group of antimicrobials designated as *antibiotics*, which are approved only for use as clinical therapeutic agents. Bacteriocins, peptides of relatively low molecular weight but, at approximately 2 to 8 kDa, larger than antibiotics, are bactericidal against gram-positive bacteria. In addition to bacteriocins, the terms *lantibiotics* and *bacteriocin-like inhibitory substances* are used to describe some of these compounds, which are quite potent antimicrobials. For example, one molecule of colicin may be sufficient to inactivate a bacterial cell (Ray and Daeschel, 1994).

The classic colicin A, produced by *Escherichia coli*, binds to specific receptors and forms voltage-dependent channels in membranes, thereby destroying the cell's energy potential and increasing membrane permeability and ion leakage (Braun et al., 1994; Parker et al., 1989). Most bacteriocins studied seem to interact specifically with membranes to cause pore formation, leakage, and dissipation of the proton motive force, which is a common mode of action for other antimicrobials such as acids and antimicrobial proteins (Bruno, 1994; Montville and Bruno, 1994). Available knowledge, especially of nisin (Figure 3.28), indicates that amphipathic bacteriocin molecules of α -helical and α -sheet structure may produce in cell membranes poration complexes resulting in leakage of cellular components and loss of membrane electrical potential (Muriana, 1996). Certain bacteriocins, however, can enter the cell and act as nucleases degrading ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). The relative largeness, the peptide nature of bacteriocins, and their binding to components of foods, may limit their potential interaction with microbial cells. These limitations may explain the relatively weak activity of bacteriocins against microorganisms in foods (Muriana, 1996).

The genes for bacteriocin production, secretion, and immunity usually are encoded on a plasmid, and production instability may occur in foods. Under adequate growth conditions, the bacteriocin gene expression is repressed by a protein repressor encoded by a chromosomal gene. When cells are stressed, the survival mechanism response triggers proteolytic activity, destroying the repressor and depressing bacteriocin transcription. Environmental conditions affect expression, including nutrient composition of the growth medium (de Vos et al., 1995; Hurst, 1983; Klaenhammer, 1988; Sahl et al., 1995), pH, and cell culture growth phase. Strains are being developed for

constitutive production of bacteriocins, or proteins may be produced in bulk and added to food as a concentrate (Barefoot et al., 1992).

During the late 1980s and early 1990s, the properties and the effectiveness of bacteriocins from food-grade fermentation starter culture bacteria (especially lactic acid producing bacteria) in culture media and their potential uses as biopreservatives in foods (Muriana, 1996) were studied intensively. Although most reported research has been conducted in laboratory culture media some trials have evaluated bacteriocin use in foods. These studies generally have yielded variable results, ranging from no inhibition to moderate or extensive antimicrobial activity, depending on product, bacteriocin, and application condition (Muriana, 1996).

Inhibition of sensitive microorganisms by bacteriocins in foods generally is moderate and often is followed by growth. Thus, target organisms may be developing resistance, or bacteriocin quantity may be inadequate to react with all target cells (Muriana, 1996). Antimicrobial activity of bacteriocins in foods

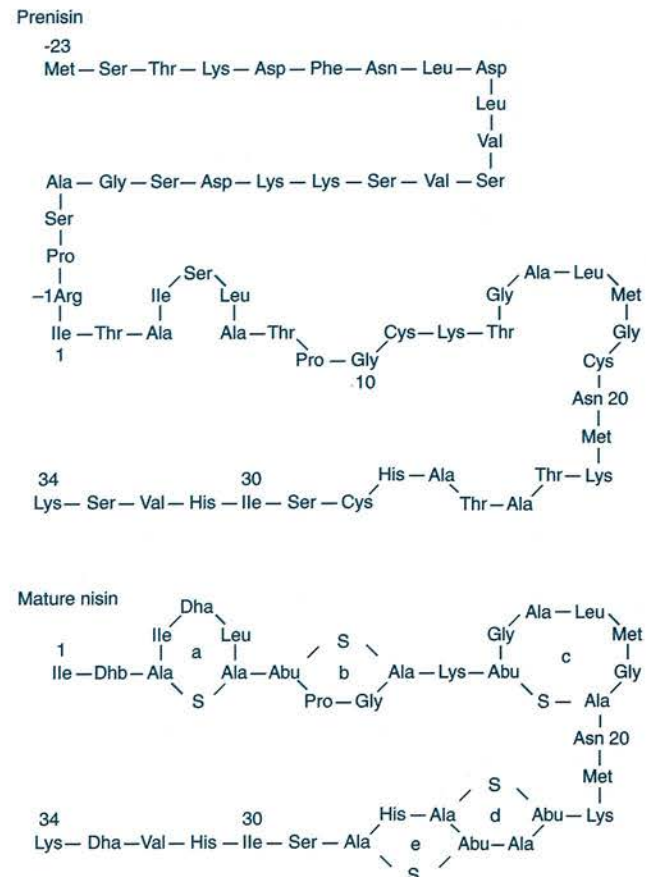


Figure 3.28. Nisin, a bacteriocin that acts against bacteria is approved for use in process cheese products (from Delves-Broughton and Gasson, 1994).

is affected by amount of bacteriocin, types and number of microorganisms, condition of application, interaction/inactivation by food components, and pH and temperature of product.

It is of concern that regular application of bacteriocins in foods may lead to development of microbial resistance to bacteriocins, as occurs with nisin-resistant variants of *Listeria monocytogenes* (Rekhif et al., 1994; Xintian and Daeschel, 1993, 1995). Development of spontaneous resistance may result from alterations or mutations associated with the cell surface or with constituents of the cell membrane. True bacteriocin resistance may be observed as a result of proteolytic activity (nisinase) of *innate*, or gene encoded, immunity, or of inherent resistance (the natural interaction of bacterial cells with bacteriocin). Gene-encoded immunity is of concern because of the potential for genetic transfer of bacteriocin resistance to pathogenic microorganisms (Muriana, 1996).

The most extensive research in foods has evaluated nisin produced by *Lactobacillus lactis* subsp. *lactis*. Nisin generally is a relatively broad-spectrum lantibiotic-type bactericidal to many gram-positive bacteria, including the pathogens *Clostridium botulinum*, *Listeria monocytogenes*, *Micrococcus*, *Staphylococcus aureus*, *Corynebacterium*, and *Mycobacterium* (De Vuyst and Vandamme, 1994; Hoover, 1992; Hurst, 1983). A combination of nisin with sorbate was active against *L. monocytogenes* in broth, but the listericidal effect was not enhanced when the two compounds were combined with lactate (Buncic et al., 1995). By its use in combination with EDTA or other compounds such as lactate, citrate, phosphate, and ethyl maltol, nisin may be made active against *Salmonella* and other gram-negative pathogens such as *Escherichia coli* and *E. coli* O157:H7 (Cutter and Siragusa, 1995a, b; 1996a, b; Schved et al., 1996; Stevens et al., 1991); nisin does not, however, inhibit *C. botulinum* in temperature abused cured meat products (McMullen and Stiles, 1996). Inhibition of bacteria by nisin in meats usually is limited and affected by storage temperature.

Nisin initially was suggested as a food preservative in 1951 by Hirsch, who found that nisin-producing starter cultures could prevent gas formation and late-blowing of cheese (Hurst, 1983) (Figure 3.29). Since this original work, nisin has been tested for antimicrobial efficacy in a wide variety of foods (Delves-Broughton, 1990). The most promising uses are in processed cheeses, dairy desserts, milk, canned foods, nitrite-cured meats, and alcoholic beverages (Delves-Broughton, 1990; Hurst, 1981; Ray and Daeschel, 1992; Zottola et al., 1994). The factors affecting its

activity in foods were reviewed by Hurst and Hoover (1993). Nisin preparations have been approved for use in process cheese products (Ronk, 1988), and approval for other applications may occur in the future.

Like nisin, certain bacteriocins can be not only active against gram-positive bacteria but also bactericidal under certain conditions, even to gram-negative bacteria and yeasts (Ray and Daeschel, 1992). Bacteriocins formed by starter culture bacteria retain bactericidal activity over a wide range of temperatures and pH values, and for relatively long periods of storage (Ray and Daeschel, 1994).

The lactococci produce, in addition to nisin, these bacteriocins: lacticins, lactococcins, dricin, diplococcin, and lactostrepcins (Hoover, 1992; McDougall et al., 1994). Nisin is the most thoroughly studied bacteriocin, however, and the only one approved for use as a food additive. Lacticin 481 inhibited strains of *Lactococcus*, *Lactobacillus*, and *Clostridium tyrobutyricum*; lactococcin was bactericidal against strains of *Clostridium butyricum*, *Streptococcus thermophilus*, and *L. helveticus*. Diplococcin was active only against strains related to the producing strain of *Lactococcus lactis* subsp. *cremoris* 346 and is sensitive to proteolytic enzymes and to heat. Lactococcin A also as effective against related species of bacteria (Ray and Daeschel, 1994).

Genes for nisin biosynthesis can be transferred to cultures other than the sucrose-fermenting strains of *Lactococcus lactis* subsp. *lactis*, which can be taken advantage of in development of improved lactic acid bacterial starter cultures. One concern, however, is that nonproducing strains in mixed cultures may be inhibited by nisin; this disadvantage could be avoided by the introduction of nisin resistance genes in starter cultures. Protein engineering efforts also may lead to an understanding of the relation between molecular structure and antimicrobial activity, stabil-

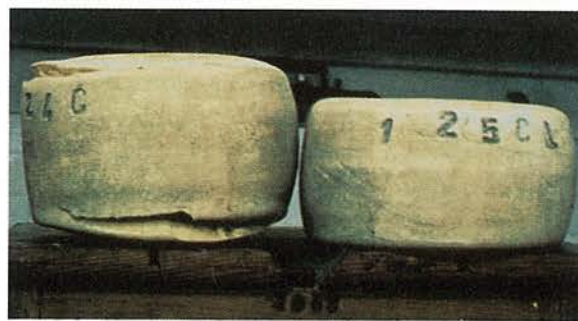


Figure 3.29. Example of gas formation and late-blowing of cheese. Photograph courtesy of Eric A. Johnson, University of Wisconsin-Madison.

ity, and specificity, as well as to the development of variants with increased activity, host range, and stability (Delves-Broughton and Gasson, 1994).

The bacteriostatic or bactericidal action of nisin against vegetative cells of bacteria is applied on the phospholipid component of the cytoplasmic membrane, where it acts as a membrane-depolarizing agent causing cell membrane leakage (Abee et al., 1994a, b; Demel et al., 1996; Winkowski et al., 1996). Nisin probably breaks the membrane permeability barrier of sensitive bacteria such as *L. monocytogenes* through pore formation rather than nonspecific detergent-like membrane destabilization effect (Winkowski et al., 1994). Gram-negative bacteria are sensitized to nisin activity upon disruption of their cell wall by chelating agents such as EDTA or by sublethal injuries caused by acid, heat, or freezing (Blackburn et al., 1989; Cutter and Siragusa, 1995a, b; 1996a, b; Kalchayanard et al., 1992). When used against spore-forming bacteria, nisin should be present in sufficient quantities to provide the residual amount needed for continued bacteriostatic action against spores. In addition, nisin in small amounts increases sensitivities of spores to heat and allows less heat treatment to inactivate spores (Hoover, 1992; Rao and Mathur, 1996). Nisin action against bacterial spores is enhanced after injury by heat (Delves-Broughton and Gasson, 1994). Nisin also inhibits bacterial spore outgrowth; in vegetative bacterial cells, it acts as a surfactant absorbed strongly on the plasma membrane, where it disrupts functioning by inactivating vital sulfhydryl groups. In addition, nisin blocks peptidoglycan formation and cell wall synthesis (Hoover, 1992). The antimicrobial action of nisin is influenced by factors such as pH, sodium chloride concentration, and storage temperature (Thomas and Wimpenny, 1996). Yeasts and molds generally are resistant to nisin activity (Delves-Broughton and Gasson, 1994).

The *Leuconostoc* species of lactic acid bacteria produce the bacteriocins mesenterocin 5, leuconosin S, leuconosin A-UAL187, and leuconosin Lcm1. Mesenterocin 5, with a molecular mass of 4.5 kDa, is resistant to heat treatment but is sensitive to the enzyme pronase and to chloroform. At relatively high concentrations, the compound is effective against *Listeria* spp. but ineffective against lactic acid bacteria and related species including *Staphylococcus aureus*, *Enterococcus*, *Micrococcus*, and *Brochothrix thermophacta* (Delves-Broughton and Gasson, 1994; Hoover, 1992; Stiles, 1994a, b).

Leuconosin S, produced by *Leuconostoc paramesenteroides*, is inactivated by proteolytic enzymes and

by α -amylase but is stable at 60°C for as long as 30 min. Active against *Listeria monocytogenes* as well as against *Staphylococcus aureus*, *Clostridium botulinum*, and even gram-negative *Yersinia enterocolitica*, the compound is active at pH values from 5.7 to 7.6 and exerts its effect by disrupting the proton motive force. Leucocin is produced by *Leuconostoc gelidum* and inhibits related lactic acid bacteria and *Listeria monocytogenes* (McMullen and Stiles, 1996; Ray and Daeschel, 1994). Leucocin A is produced by *Leuconostoc gelidum* UAL187 at refrigeration temperatures, which would make it useful in the preservation of foods kept in cold storage. When sucrose is present, however, the latter organism also produces both polysaccharides such as dextran and carbon dioxide gas, which may be undesirable in various foods (McMullen and Stiles, 1996). Leucocin B-Ta11a was produced by *Leuconostoc carnosum* Ta11a, isolated from processed meat, and found to be heat stable but sensitive to proteolytic enzymes (Felix et al., 1994). Carnocin 54, produced by *Leuconostoc carnosum* LA54A, was lethal against *L. monocytogenes* (Schillinger et al., 1995). Mesenterocins and dextranocins are produced by *Leuconostoc mesenteroides* and *dextranicum*, respectively (Sudirman et al., 1994).

The meat and vegetable fermenters *Pediococcus pentosaceus* and *P. acidilactici* produce pediocin A and pediocin AcH, respectively. The latter organism also produces pediocin PA-1 (Figure 3.30). The host range of pediocin A is relatively wide and includes strains of *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Staphylococcus*, *Listeria*, and *Clostridium*. Pediocin AcH is inhibitory against *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Micrococcus*, *Bacillus*, *Staphylococcus*, *Clostridium*, *Listeria*, and *Brochothrix*. It also is effective against gram-negative bacteria such as *Escherichia*, *Salmonella*, *Yersinia*, *Aeromonas*, and *Pseudomonas* after sublethal pretreatment of cells with heat, freezing temperatures, or weak acids (Ray and Daeschel, 1992, 1994).

Inactivation of gram-positive bacteria by pediocin AcH occurs through destabilization of the cytoplasmic membrane functions. Resistant gram-positive bacteria allow absorption of pediocin AcH on the cell wall but do not penetrate to cause cell destabilization or inactivation. In contrast, gram-negative bacteria lack receptors for pediocin AcH, which can penetrate the cell wall only after damage of the outer membrane by sublethal treatments. Production of pediocin AcH is linked with an 8.9 kilobase (kb) plasmid, the 186 nucleotides of which code for a protein of 62 amino acids, with a final active pediocin AcH molecule of 44 amino acids. Pediocin AcH inhibits gram-positive bac-

teria in food systems (Ray and Daeschel, 1994).

Pediocin PA-1 is produced by *Pediococcus acidilactici* PAC 1.0 and has the same 44 amino acid sequence as pediocin AcH. The two disulfide bonds of pediocin PA-1 give it a coiled conformation, which makes it stable at temperatures between -20 and 100°C, but it loses activity after treatment with proteolytic enzymes. Strains affected by pediocin PA-1 belong to *Listeria monocytogenes*, *Pediococcus acidilactici*, *P. pentosaceus*, *Lactobacillus plantarum*, and *Leuconostoc dextranicum*. Although difficult to purify, pediocin PA-1 has shown antimicrobial activity in products such as cottage cheese and fresh beef. As with other bacteriocins, however, initial inactivation or inhibition of bacteria by pediocin PA-1 may be followed by multiplication during storage (Ray and Daeschel, 1994).

Lactobacillus spp. produce bacteriocins such as lactocins B and F, lactocins 27 and S, plantaricin F and SAG brevicins, caseicin 80, acidocin A, helveticin J, plantacin B, and sakacin A (Hoover, 1992). *Lactobacillus acidophilus* N2 produces lactacin B, a heat-resistant bactericidal protein sensitive to proteolytic enzymes (Barefoot et al., 1994). Lactacin F is produced by *L. acidophilus* 88, which seems to be composed of 57 amino acids. Acidophilicin A is a product of *L. acidophilus* LAPT 1060, which is sensitive to heat and proteolytic enzymes. *Lactobacillus brevis* produces

heat stable but proteolytic enzyme-sensitive brevicin 37, which inhibits several lactic acid bacteria. Caseicin 80 is produced by *L. casei* B80 and is active only against *L. casei* B109. Curvacin A is produced by *Lactobacillus curvatus* LTH1174 and acts against *L. monocytogenes* and *Enterococcus faecalis* (Tichaczek et al., 1993). Lactacins A and B are produced by *Lactobacillus delbrueckii* subsp. *lactis* JCM 1106, 1107, and 1248, but their host range also is narrow and is lost after heat treatment (60°C, 10 min) or exposure to proteolytic enzymes. Lactacin F is produced by *Lactobacillus johnsonii* VPI 11088 and affects *Lactobacillus* and *Enterococcus* spp. (Allison et al., 1995). Other *Lactobacillus* strains producing bacteriocins are *L. fermenti*, *L. gasseri*, *L. helveticus*, *L. plantarum*, *L. reuterii*, and *L. sakei* (Ray and Daeschel, 1994). *Lactobacillus plantarum* BF001, isolated from chilled processed catfish, produced plantaricin F, which inhibited a wide range of foodborne pathogenic bacteria (Fricourt et al., 1994).

Strains of the dairy *Propionibacterium* species also have been found to produce bacteriocins (Hoover, 1992). *Propionibacterium thoenii* forms propionicin PLG-1, which inhibits strains of *Propionibacterium*, lactic acid bacteria, and strains of the gram-negative *Aeromonas* and *Yersinia*. In contrast, strains of *Staphylococcus*, *Clostridium*, *Bacillus*, and *Salmonella* were not inhibited. Jensenin Gi, produced by *P. propionibacterium* subsp. *jensenii* P126, is destroyed by proteinases and partly by high (100°C) temperatures (Grinstead, 1994; Ray and Daeschel, 1994). *Carnobacterium piscicola* and *C. divergens* produce carnosin and divergicin, respectively (McMullen and Stiles, 1996; Pilet et al., 1995; Saucier et al., 1995).

According to Ray and Daeschel (1994), a bacteriocin, to be suitable as a food preservative, should have the following characteristics: (1) it should be effective against a broad spectrum of spoilage and pathogenic bacteria, including spore formers; (2) it should destroy most cells in a population of a sensitive strain; (3) it should maintain its activity in variable food environments; (4) it should not affect food quality; (5) it should be cost-effective; and (6) adequate information should be available to support its approval by regulatory agencies.

Lastly, the discovery of bacteriocins in food fermentative bacteria indicates that organisms competing in mixed populations for resources and space have evolved mechanisms for inactivating competing organisms. One would predict that other food fermentative organisms such as yeasts and molds also produce antimetabolites, but these have not been investigated.

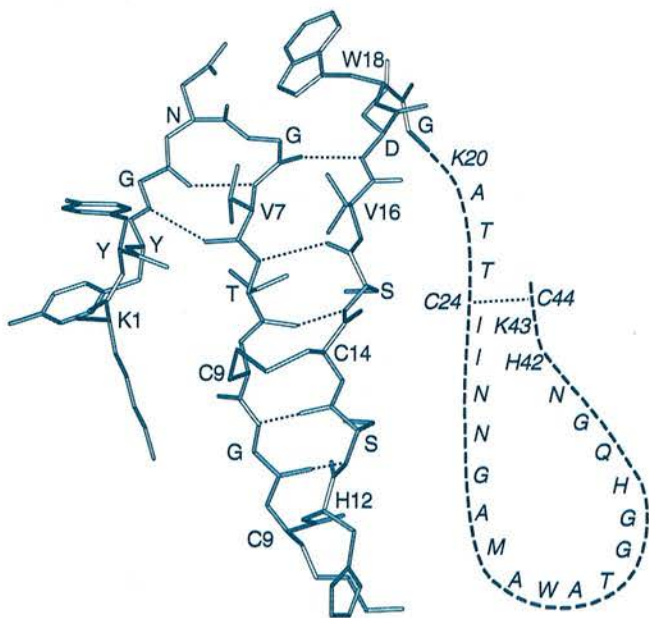


Figure 3.30. Meat and vegetable fermenting bacteria produce pediocin PA-1, which is effective against certain pathogenic bacteria (Chen et al., 1997).

Killer Toxins

Strains of *Saccharomyces cerevisiae* first were found to secrete a protein inactivating a limited number of strains of related yeasts (Bevan and Makower, 1963). In other genera of fungi, a broader range of such activity has been found, and the compounds involved are called *killer toxins*. The phenomenon of killer activity is known now to occur widely in many genera of yeasts. Killer strains of *Saccharomyces* produce proteins or glycoproteins killing sensitive strains, usually by perturbation of the plasma membrane although the killer toxin from *Kluyveromyces lactis* was reported to inhibit DNA synthesis (Martinac et al., 1990). Killer activity of different yeasts in the genus *Saccharomyces* depends on the presence of two double stranded RNA plasmids. In *K. lactis*, the lactose fermenting yeast, two plasmids are responsible for killer activity.

The activity of killer strains, inasmuch as it usually is narrow and effective against closely related yeasts, resembles the narrow spectrum of bacteriocin activity. Recently, a killer strain of the yeast *Zygosaccharomyces bailii* that produced a killer toxin lethal to sensitive strains of *Saccharomyces cerevisiae* and *Candida glabrata* was isolated (Radler et al., 1993). The killer protein was purified and had a molecular mass of approximately 10 kDa, similar to the range of 10 to 20 kDa found for other killer toxins. Killer toxins are acidic molecules with isoelectric points close to pH 4. These toxins attach to a mannoprotein on the cell wall or to a cell wall receptor containing a α -1,6-D-glucan and then disrupt the plasma membrane, causing ion loss and cell death (Dillon and Cook, 1994).

The role of killer toxins has been investigated in wine and beer fermentations. The presence of killer strains in grape musts may contribute to sluggish or stuck fermentation (Jacobs and van Vuuren, 1991), which typically has been attributed to nitrogen deficiency. Surveys of yeast microflora of grapes and other fermenting foods have detected the presence of killer yeasts (Rosini, 1983). Killer yeasts in the genera *Debaryomyces*, *Hansenula*, *Candida*, and *Pichia* have been detected in various fermenting foods including miso, soy sauce, and salted vegetables (Dillon and Cook, 1994; Suzuki et al., 1989a).

Defensins

An intense subject of antimicrobial research during the late 1980s and 1990s has been the nature and the action mode of antimicrobial defensins (Lehrer et al., 1991). Defensins comprise a family of small molecular weight peptides of 29 to 34 amino acids. They are cationic, cysteine-rich peptides believed to kill by

insertion into the membrane of microorganisms, thereby forming pores and causing increased permeability to ions (Kagan et al., 1990). Defensins have been found in humans and certain other mammals and have antibacterial, antifungal, and antiviral activities *in vitro*. They are synthesized in abundance in granules on neutrophils and macrophages and in cells of the small intestine (Lehrer et al., 1991). They have extremely potent antimicrobial activity and seem to be potential agents for food preservation.

Magainin 2 is a peptide containing 23 amino acids and it was originally isolated from the African clawed frog (*Xenopus laevis*) (Zasloff, 1987). This peptide has antimicrobial activity against gram-positive and gram-negative bacteria and fungi. Abler et al. (1995) tested magainin 2 and magainin 2 amide (alandine residues at positions 8, 13, and 18) against 13 food-borne pathogens including six strains of *Salmonella*, two strains of *Vibrio cholerae*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Magainin, at 100 $\mu\text{g/mL}$, had bactericidal activity against two strains of *Salmonella* and *cholerae*, but all strains recovered to the level of the controls by 24 hr. In contrast, magainin 2 amide at 3 to 50 $\mu\text{g/mL}$ was bactericidal to all strains tested, with log reductions of 7 to 8 at 24 hr. Activity of magainin 2 amide was greatest at temperatures above 20°C and was reduced significantly by the presence of bovine serum albumin. The mechanism proposed for magainins involves their ability to form the amphipathic helix structure, which creates ion channels in the cell membrane and disrupts membrane potential, leading to inactivation (Abler et al., 1995).

Antimicrobial Agents in Insects

The successful colonization and the great diversity of insects is, at least in part, due to their resistance to microbial attack (Dillon, 1994). Insects have developed several defense mechanisms, including (1) the cuticle, which, in addition to being a physical barrier, contains chemical antimicrobial agents of unknown importance, such as short-chain fatty acids; (2) the gut, which contains physical barriers or physicochemical factors—e.g., pH, water activity, limited oxygen level, potentially the normal gut microflora, and food derived antimicrobials including chlorogenic acid, caffeic acid, and quinones—acting as inhibitors of microbial growth; (3) antimicrobial insect secretions such as skatole, hydrogen peroxide, phenols, phenylacetic acid, and acid mucopolysaccharide; and (4) components of the haemolymph, including lysozyme, phenoloxidase, immunoglobulin-like haemolin, lec-

tins, agglutinins, and inducible peptides (Boman and Hultmark, 1987; Dillon, 1994).

Insects are known for their inducible immune system, which is based on lysozyme and the secretion of antibacterial peptides into the haemolymph (Boman and Hultmark, 1987). Several known families of antibacterial peptides (Table 3.8) are present in insects, including the cecropins and attacins (Casteels, 1990; Dillon, 1994). They are small, with a molecular weight of approximately 4 kDa, strongly basic proteins with potent bactericidal activity and are present in many insect orders. These peptides have a common cytolytic region also present in certain toxic enzymes including hemolysins, cadiotoxins, myotoxins, and melittin (the main component of bee venom). They induce a rapid lysis of cells of gram-positive and gram-negative bacteria (although not of mammalian cells), probably by disrupting membranes directly and causing active transport loss and ATP reduction (Dillon, 1994). Cecropins and attacins likely work in synergy with lysozyme (Boman and Hultmark, 1987; Engstrom et al., 1984). Possible applications of insect antibacterial peptides as therapeutics and in food and feed have been suggested (Casteels, 1990).

Table 3.8. Antibacterial peptides found in insects (from Dillon, 1994).

Class ^a	Spectrum of activity
Abaecin (34)	Moderate activity against gram-negative and gram-positive bacteria. Contains ten proline residues.
Apidaecin (18)	Highly bacteriostatic towards gram-negative bacteria. Non-helical structure contains six proline residues.
Attacin (185)	Highly bacteriolytic against growing cells of a few gram-negative species, moderate activity against a wider range. Also sarcotoxin II.
Cecropin (36)	Highly bacteriolytic against a broad spectrum of gram-positive and negative bacteria. Also sarcotoxin I.
Coleopteracin (74)	Glycine-rich, active against gram-negative bacteria.
Defensin (40)	Highly active against gram-positive bacteria. Contains three disulfide bonds. Most widespread group.
Diptericin (82)	Narrow activity spectrum against growing cells of some gram-negative bacteria. High glycine content. Also sarcotoxin III.
Royalisin (51)	Constitutive in the royal jelly of bees. Highly active against gram-positive bacteria. Contains three disulfide bonds. Structurally similar to defensins.

^aNumbers in parentheses are amino acid residues.

That eukaryotic cells are resistant to cecropins suggests that they attack bacteria specifically. This specificity likely depends on the interaction with the target membrane (Steiner et al., 1981, 1988). Cecropins have been found in the small intestine of pigs (Lee et al., 1989); they therefore may be involved in the regulation of bacterial colonization of the gut. Casteels (1990) suggested that antibacterial peptides could be cloned and expressed in desirable organisms such as lactic acid bacteria. It also was suggested that expression of the peptides in plants could enhance their defense systems to prevent infection or postharvest spoilage. It is not known, however, whether normal growth would be impaired.

Diptericins, also inducible antibacterial enzymes but unrelated to cecropins, are active only against growing cells of a limited number of species. Attacins, which are larger molecules (20 kDa) than cecropins, are quite effective against growing cells of a few gram-negative bacterial species and are moderately effective against a greater number of bacteria. Defensins are composed of approximately 40 amino acid residues with three disulfide bonds and are effective against gram-negative bacteria. They have amino acid sequences similar to those of vertebrate defensins. Abaecin, royalisin, and apidaecins are found in bees and may contribute to the antimicrobial properties of honey.

Of these other insect immune peptides, attacins, apidaecins, and abaecin have not been shown to have lytic activity, but attacins and abaecin seem to have limited activity against bacteria. The apidaecins are strongly active against gram-negative bacteria, which is not due to lytic activity, and against *Salmonella* serovars and *Shigella* spp. (Dillon, 1994).

As indicated, insects contain a large pool of known or unidentified chemical antimicrobials. In addition, they may be the source of antimicrobial compounds derived from microorganisms found in association with insects (Dillon, 1994). These complex associations, through the numerous potent antimicrobial agents produced by the microorganisms, may be very important to insect survival. Active substances investigated, isolated, or even patented include hydroxyl- and acetoxyl-indole derivatives, 4-ethyl- and 4-isopropyl-3,5-dihydroxy-*trans*-stilbenes, dithiopyrrolones, xenorhabdins, benzopyran derivatives, xenocoumacins, phenylacetic acid, phenylacetaldehyde, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, hydroquinone, bacteriocins, and lysozyme (Dillon, 1994).

Although seemingly promising, the potential use of insects as sources of antimicrobial food preserva-

tives has not been explored. A practical example is the antimicrobial system introduced by the honey bee in honey. Royal jelly contains the peptide royalisin and hydroxydecanoic acid, which provide complementary respective antimicrobial activities against gram-positive and gram-negative species. Ethanol extracted *propolis*, a resinous material produced by honey bees, extended the shelf-life of meat products more than potassium sorbate did (Han and Park, 1996). Honey also can be a source of contamination, however (Dillon, 1994; Snowdon and Cliver, 1996).

Miscellaneous Polypeptides

Bovine milk and egg albumen contain proteins that bind vitamins strongly. Avidin, a glycoprotein in egg white, binds biotin very strongly; this protein exhibits the highest known affinity for a ligand (K_a approximately $10^{15}M^{-1}$) (Livnah et al., 1993). Avidin and its complex with biotin have marked stability to heat or degradation by proteolytic enzymes. The enzyme may inhibit biotin-requiring organisms such as yeasts, *Lactobacillus* spp., and *Clostridium* spp., but the natural antibacterial role of the protein has not been demonstrated. Similarly, milk contains proteins binding the vitamins B₁₂ and folic acid strongly, and the vitamin binders may restrain the growth of specific organisms in these foods (Reiter, 1978).

The entire complement of riboflavin in egg albumen occurs complexed as the flavin moiety of ovoflavoprotein, a glycoprotein with a molecular weight of 32 kDa. The protein may contribute to inhibition of microbial growth by sequestration of riboflavin, particularly when combined with starvation for nutrients such as iron and biotin, but this possibility has yet to be proved.

By virtue of their capacity to bind water and to lower water activity, food proteins have been considered as bacterial growth inhibitors in foods. Traditionally, water activity has been lowered by use of salts, alcohols, or other humectants, and by freezing. Certain proteins also bind substantial quantities of water. Kumosinski et al. (1988) reported that casein suppresses water activity more than lactoglobulin does. Addition of casein at 10 times its concentration in milk lowered the water activity at 4°C to 0.95, which is sufficiently low to inhibit the growth of Group II *Clostridium botulinum* and *Salmonella*. The antimicrobial forms of casein are the peptides casein- α_{s2} -165-203 and casein- α_{s2} -166-203; these are known as α_{s2} -casobiotics (Zucht et al., 1996).

Basic molecules including aprotinin, protamine, and histones, that have proteinase inhibitory activi-

ty or that bind nucleic acids also have antibacterial properties. Miller et al. (1942) showed that gram-positive bacteria were inactivated whereas gram-negative bacteria were sensitized to inactivation after exposure to protamine, histones, or basic proteins from salmon sperm. Pellegrini et al. (1992) reported that the bactericidal activities of aprotinin, a protease inhibitor, and lysozyme against gram-positive as well as gram-negative bacteria depended primarily on the basic character of molecules. These researchers suggested that bactericidal activity was due to cationic and hydrophobic properties possibly promoting autolysis. These basic proteins may have application in sensitizing bacteria to other antimicrobials in foods.

Bjorck et al. (1989) designed interesting peptide derivatives that mimicked naturally occurring proteinase inhibitors. A tripeptide derivative had antibacterial activity against a large number of bacterial strains belonging to 13 different species. The mode of action was probably inhibition of essential protease activity. This approach could lead to construction of new agents for control of microorganisms in foods.

Miscellaneous Microbial Metabolites with Antimicrobial Activity

Introduction

Many microorganisms involved in fermentation produce, in addition to the bacteriocins and related compounds previously described, metabolites with demonstrated antimicrobial activity against other microorganisms. These metabolites include ethanol produced by yeasts and heterofermentative bacteria, natamycin produced by a *Streptomyces* spp., and diacetyl and hydrogen peroxide produced by lactic acid bacteria. Many of these compounds not only are natural antimicrobials but also are allowed, by the *Code of Federal Regulations*, as additives for various purposes in foods.

Throughout the world, fermentations, especially of the lactic acid type, are critical in the preservation of foods (Johnson, 1991). These fermentations are based on (1) uncontrolled, spontaneous predominance of the desirable bacteria; (2) use of inoculum from a successful fermentation, i.e., *back-slopping*; or (3) use of purified starter culture preparations. These fermentations are a biocontrol system inhibiting undesirable microorganisms potentially involved in spoilage or foodborne illness (Gombas, 1989). Preservation of

products is due mainly to organic acids such as lactic or acetic acid, which decrease pH (Daeschel, 1989). Other factors that may contribute to microbial inhibition include decreased activity of water, lack of oxygen, depletion of nutrients, and production of small quantities of antimicrobial substances such as bacteriocins, ethanol, hydrogen peroxide, and diacetyl (Dillon and Cook, 1994; Johnson, 1991; Lücke and Earnshaw, 1991). Certain organisms also produce propionic acid, a relatively potent antimicrobial agent. Reuterin (3-hydroxypropionaldehyde) is a low molecular weight, neutral pH, soluble, nonprotein broad-spectrum antimicrobial produced by heterofermentative *Lactobacillus reuterii* (Daeschel, 1989). Reuterin is active against all types of microorganisms, including gram-positive and gram-negative bacteria, yeasts, molds, and protozoa. The antimicrobial activity of reuterin has been verified in several foods (Dillon and Cook, 1994).

As has been emphasized, organic acids are useful inhibitors of microbial growth and serve as preservatives in various foods. The group of homofermentative lactic acid bacteria, i.e., *Lactococcus*, *Streptococcus*, *Pediococcus*, and some *Lactobacillus*, metabolizes sugars and produces mostly lactic acid whereas the heterofermentative group, i.e., *Leuconostoc*, the other *Lactobacillus* spp., converts glucose to lactic acid and equimolar amounts of acetate, ethanol, and carbon dioxide as well as, in lesser amounts, other end-products such as acetaldehyde, formic acid, diacetyl, acetoin, and 2,3-butylene glycol (Dillon and Cook, 1994).

Organic acids such as lactic, acetic, citric, fumaric, erythorbic, malic, and succinic acids also are produced by certain fermentative fungi, e.g., *Aspergillus*, *Amylomyces*, *Penicillium*, *Mucor*, and *Rhizopus*, in foods. Other products of fungi and yeasts are ethanol and carbon dioxide. Additional antimicrobial products of yeasts are glycerol, succinic acid, diacetyl, lactic acid, formic acid, acetic acid, higher alcohols, and acetoin (Dillon and Cook, 1994).

Ethanol

The value of ethanol (C_2H_5OH) as a naturally occurring antimicrobial has been recognized since the first alcoholic fermentations of fruits to produce wines (Figure 3.31), which occurred several thousand years ago. The primary natural source for ethanol in foods is the alcoholic fermentation of sugars by yeasts. Heterofermentative lactic acid bacteria, i.e., *Lactobacillus* and *Leuconostoc*, also are capable of producing ethanol; however, they generally produce less than 1%

in foods, which is below the concentration necessary for significant antimicrobial activity (Lücke and Earnshaw, 1991).

For ethanol to disinfect, water must be present (Seiler and Russell, 1991). At 95% ethanol, most vegetative cells are resistant whereas, at 60 to 75% ethanol, most microorganisms are destroyed in less than 1 min (Seiler and Russell, 1991; Shelef and Seiter, 1993). Low concentrations (less than 30%) of ethanol are rarely biocidal alone but may exert inhibitory effects (Banwart, 1989). At 8 to 11% (v/v), ethanol prevents growth of most molds and bacteria; a 15 to 18% concentration is required to prevent growth of most yeasts (Seiler and Russell, 1991). Gram-negative bacteria are more susceptible to ethanol than gram-positive bacteria are. Bacterial spores generally are resistant to the compound. Certain bacteria, including *Lactobacillus* species, *Zymomonas mobilis*—an ethanol producer, and *Escherichia faecalis*, are relatively resistant to the antimicrobial effects of ethanol (Seiler and Russell, 1991). Some osmophilic yeasts (e.g., *Saccharomyces rouxii*) also are relatively resistant.

In addition to type of microorganism, various en-



Figure 3.31. Ethanol is an effective naturally occurring antimicrobial that has been recognized since the first alcoholic fermentations of fruits to produce wines.

environmental factors influence the activity of ethanol as an antimicrobial. Increased sugar concentration and decreased water activity increase its effectiveness (Shapiro et al., 1978), but presence of organic matter decreases activity (Shelef and Seiter, 1993). Decreasing storage temperature evidently increases the antimicrobial effectiveness of ethanol (Gray, 1948).

Ethanol may enhance the effectiveness of other food antimicrobials. Splittstoesser and Stoyla (1989) found that 5 to 10% ethanol in combination with 0.00015% fumaric acid, 0.03% sorbic acid, 0.002% decanoic acid, and 100 IU of nisin inhibited the growth of *Leuconostoc oenos* in a model grape juice system. The effectiveness of antimicrobials depended greatly on initial number of bacterial cells present. Ethanol does not always improve the effectiveness of other antimicrobials, however. Parish and Carroll (1988) demonstrated that combinations of ethanol and potassium sorbate or sodium metabisulfite were only additive in their inhibitory effectiveness against *Saccharomyces cerevisiae* in a model broth system. A combination of ethanol and butyl paraben actually produced an antagonistic antimicrobial response.

Many cellular targets of ethanol have been identified, including glycolytic enzymes; peptidoglycan synthesis; DNA, RNA, and protein synthesis; protein secretion; fatty acid biosynthesis; phospholipid biosynthesis; membrane permeability; and solute uptake/ion pumping (Seiler and Russell, 1991). Many of these effects likely are secondary. Because ethanol is partly lipid soluble or, *amphophilic*, the primary site of activity is thought to be the cytoplasmic membrane. It also is thought that ethanol may have a direct effect on membranes or membrane-bound enzymes, or an indirect effect due to impairment of membrane biosynthesis (Seiler and Russell, 1991). Dissolution of ethanol in the cell membrane increases fluidity of the lipid and decreases gel-to-liquid crystalline phase transition temperature of lipids. This results in disruption of membrane organization, leakage of ions, leakage of low molecular weight solutes, and sometimes leakage of macromolecules (Ingram and Buttke, 1984; Osman and Ingram, 1985; Salgueiro et al., 1988).

Ethanol has GRAS status as a food additive in the United States (Code of Federal Regulations, 1992). It is not a specified food additive in the United Kingdom (Seiler and Russell, 1991). Ethanol is used primarily as a solvent for food flavors and colors.

Natamycin

Natamycin first was isolated in 1955 from a culture of *Streptomyces natalensis*, a microorganism found in soil from Natal, South Africa (Anonymous, 1991) (Figure 3.32). The generic name *natamycin* is synonymous with *pimaricin*, a name used in earlier literature. Natamycin is a polyene macrolide antibiotic with the formula $C_{33}H_{47}NO_{13}$ (MW = 665.7). Solubility ranges of natamycin in selected solvents include 30 to 100 mg/L for water (ambient temperature), 2 to 15 mg/L for methanol, 0.04 to 1.2 mg/L for ethanol, 0.05 to 0.12 mg/L for n-butanol, and 0.01 to 0.013 mg/L for chloroform (Brik, 1981).

Natamycin is active against nearly all molds and yeasts but has no effect on bacteria or viruses (Davidson and Doan, 1993; Gould, 1996). Most molds are inhibited at concentrations of natamycin from 0.00005 to 0.0025%. Most yeasts are inhibited at natamycin concentrations from 0.0001 to 0.0005%. Woolford (1975) found no inhibition of bacteria at pH 5.0 and natamycin concentrations of > 0.02%. In contrast, all yeasts and molds were inhibited by 0.00373 to 0.00998% at pH 5.0 or 6.0.

In addition to inhibiting fungal growth, natamycin influences mycotoxin production. Ray and Bullerman (1982) reported that 0.001% natamycin inhibited aflatoxin B₁ production of *Aspergillus flavus* by 62.0% and eliminated ochratoxin production by *A. ochraceus*. The same level of natamycin decreased penicillic acid production by *Penicillium cyclopium* and eliminated pat-

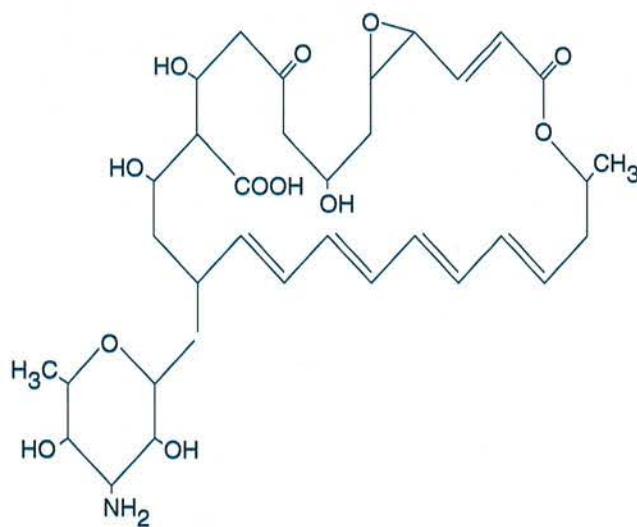


Figure 3.32. Natamycin is active against nearly all molds and yeasts.

ulin production of *P. patulum*. Gourama and Bullerman (1988) studied the effect of natamycin (0.035%) on growth and mycotoxin production (penicillic acid) by *A. ochraceus* OL24 in olive paste and found that natamycin at 25°C delayed growth initiation for 1 to 4 days and penicillic acid production by 96%. The authors concluded that natamycin could provide protection against fungal growth and mycotoxin formation in olives. Natamycin at 0.005% was effective in inhibiting *A. parasiticus* growth and toxin production on raw peanuts by 99% after 11 days (Gelda et al., 1974).

Several factors including pH, temperature, light, oxidants, and heavy metals affect the stability and antimycotic activity of natamycin. Although having no evident effect on natamycin's antifungal activity, pH does influence its stability. In the pH range of most food products (5.0 to 7.0), natamycin is very stable. Under normal storage conditions, temperature has little effect on natamycin activity when in neutral aqueous suspension. Little or no decrease in activity occurs after several days at 50°C or for a short time at 100°C (Brik, 1981). Sunlight, contact with oxidants such as organic peroxides and sulfhydryl groups, and heavy metals all adversely affect stability of natamycin solutions or suspensions (Anonymous, 1991; Brik, 1981).

The mode of action of polyene macrolides involves binding of ergosterol and other sterol groups in fungal cell membranes. Interaction of natamycin with sterols inhibits ergosterol biosynthesis, distorts the cell membrane, and causes cellular material leakage (Hamilton-Miller, 1973). Natamycin use evidently does not result in fungi with increased tolerance to the compound. de Boer and Stolk-Horsthuis (1977) found no evidence of resistant fungi in cheese warehouses in which natamycin had been used for various periods and up to several years.

Nonprotein Inhibitors from Lactic Acid Bacteria

Introduction

Preservation of fermented foods by means of lactic acid bacteria is due primarily to the production of organic, e.g., lactic and acetic, acids, the decreasing of pH, and the removal of carbohydrates as nutrient sources (Daeschel, 1989; Gilliland, 1985). In addition, as indicated, lactic acid bacteria produce substances including hydrogen peroxide, diacetyl, and bacteriocins, which have the potential to inhibit a variety of other microorganisms (Daeschel, 1989).

Diacetyl

Diacetyl, or 2,3 butandione ($\text{CH}_3\text{COCOCH}_3$), is pro-

duced by the citrate-fermenting lactic acid bacteria *Leuconostoc cremoris* and *Lactobacillus lactis* spp. *lactis* bv. *diacetylactis* (formerly *Streptococcus diacetylactis*, *S. lactis* ssp. *diacetylactis*). The compound, synthesized from pyruvate, produces a buttery flavor in fermented dairy products and in foods to which it is added for flavor. Acceptable sensory levels in dairy products range from 0.0001 to 0.0007% (Lücke and Earnshaw, 1991; Sandine et al., 1972). The compound has long been known to have antimicrobial activity.

Jay (1982a) extensively studied the antimicrobial activity of diacetyl against a number of strains of gram-positive and gram-negative bacteria, yeasts, lactic acid bacteria, and pseudomonads. He reported that 0.02% diacetyl inhibited yeasts and gram-negative bacteria, 0.03% inhibited nonlactic gram-positive bacteria, and > 0.05% inhibited lactic acid bacteria. *Pseudomonas* spp. were inhibited by 0.03% diacetyl. Several strains of *Clostridium* were not inhibited on plated agar media in anaerobic chambers but were inhibited by 0.0688% diacetyl in thioglycollate broth. Effectiveness of diacetyl increased as pH decreased from 8.0 to 5.5. Greatest inhibition was achieved in plate count agar and least inhibition in broth or cooked meat medium. Differences between culturing media were attributed to composition, inoculum size, or pH. Acetate, Tween 80, and glucose all were antagonistic to the antimicrobial activity of the compound. Jay (1982b) added diacetyl to ground beef at 0.04% and stored it at 5 to 7°C for 8 days. There was no increase in aerobic plate count or gram negative count for the storage period. In contrast, aerobic plate count increased approximately three logs in control ground beef during the same period.

Gupta et al. (1973) confirmed that diacetyl was most effective against gram-negative bacteria. Motlagh et al. (1991) tested diacetyl at 0.0344% in tryptic soy broth against three strains of *Listeria monocytogenes*, *Salmonella anatum*, *S. typhimurium*, *Yersinia enterocolitica*, *Escherichia coli* serotypes O18 and O157:H7, and two strains of *Aeromonas hydrophila*. The compound was not effective against *L. monocytogenes* but caused a slight decrease in viable cells of all gram-negative strains after 24 hours at 4°C. The greatest activity was demonstrated against *Y. enterocolitica*, the number of which decreased by 2.3 logs after 24 hours.

Whereas diacetyl is GRAS, the effective antimicrobial concentration (greater than 0.02%) is greater than that needed to play a major role as a natural preservative (approximately 0.0002 to 0.0007%) or as an added biopreservative (Daeschel, 1989; Gupta et al., 1973; Motlagh et al., 1991). Its volatility also would

limit its usefulness as an added biopreservative (Jay et al., 1983). Its role in the combination of inhibitors produced by lactic acid bacteria is, however, potentially important. A proposed mechanism by which diacetyl inhibits microorganisms is through binding of arginyl residues on proteins (Jay et al., 1983). Because of their lack of periplasmic binding proteins and larger amino acid pools, gram-positive bacteria may be more resistant to diacetyl (Shelef and Seiter, 1993).

Hydrogen Peroxide

The oxidizing, bleaching agent hydrogen peroxide has been recognized as an antimicrobial since its discovery by Thenard in 1818 (Cords and Dychdala, 1993). Both purified and naturally-produced hydrogen peroxide have potential uses as antimicrobials associated with foods. Hydrogen peroxide is approved as an antimicrobial for direct addition to dairy products and starch. It also is used to sterilize food packaging materials and as a sanitizer of surfaces coming into contact with food. Naturally-occurring hydrogen peroxide produced by lactic acid starter culture microorganisms contributes to preservation of some dairy products. As a reactant or a product of certain enzymatic reactions, hydrogen peroxide has potential to extend shelf-life or to preserve.

The antimicrobial activity of hydrogen peroxide depends on microorganism, microbial load, hydrogen peroxide concentration, exposure temperature, exposure time, and pH. The compound is active against bacteria, molds, yeasts, and viruses. It generally is especially effective against anaerobes and facultative anaerobes because they lack catalase (Lipman and Owen, 1943). Gram-negative bacteria generally are more susceptible to hydrogen peroxide than gram-positive bacteria are. Nambudripad and Iya (1951) and Nambudripad et al. (1949) found that *Escherichia coli* and *Enterobacter aerogenes* were destroyed completely by 0.05% hydrogen peroxide in 10 to 30 min at 37°C. In contrast, *Lactobacillus lactis*, *L. bulgaricus*, and *Bacillus megaterium* were significantly more resistant. Hydrogen peroxide at 0.0495% destroyed 7.3 logs of *Listeria monocytogenes* at 15°C in sterile milk (Dominguez et al., 1987).

In mixed culture with *Staphylococcus aureus* and *Streptococcus faecalis* in sterile milk or with natural milk microflora in raw milk, however, *Listeria monocytogenes* was significantly more resistant to the same concentration of hydrogen peroxide. Aerobic bacterial spores (*Bacillus* spp.) are more resistant to the effects of hydrogen peroxide than spores of *Clostridium* spp. are (Setlow and Setlow, 1993; Stevenson and Shafer, 1983). Hydrogen peroxide was sporicidal at 10

to 35% (Stevenson and Shafer, 1983; Turner, 1983). The *D-values*, i.e., the times or dosages required to decrease the population of a microorganism by 90%, using hydrogen peroxide (25.8%) at 24°C for certain microorganisms (*D-values* in parentheses) are as follows: *B. subtilis* (7.3 min), *B. coagulans* (1.8 min), *B. stearothermophilus* (1.5 min), *Clostridium* spp. (0.8 min), and *Staphylococcus aureus* (0.2 min) (Toledo et al., 1973). *Heat shocking* of the spores, i.e., exposing to sublethal heat for spore activation before treatment, decreased their resistance. Wet spores were more resistant than dry spores.

Later work by Leaper (1984) demonstrated that dry spores of *Bacillus subtilis* actually were more resistant than wet spores to the effects of 11.8 to 41.3% (w/v) hydrogen peroxide. As might be expected, the higher the concentration of hydrogen peroxide, the more rapid the inactivation (Swartling and Lindgren, 1968). Concentrations of 0.001 to 0.1% at room temperature inhibit the growth of most bacteria and fungi (Cords and Dychdala, 1993). At concentrations of 0.1% and higher, hydrogen peroxide is bactericidal and fungicidal (Cords and Dychdala, 1993). Increased temperature (Swartling and Lindgren, 1968; Toledo et al., 1973) and decreased pH (Curran et al., 1940) also are correlated with increased activity of hydrogen peroxide. Certain inorganic ions, especially copper, increase its antimicrobial activity (Stevenson and Shafer, 1983).

Certain lactic acid bacteria are capable of producing hydrogen peroxide through the actions of pyruvate oxidase on pyruvate, L-lactate oxidase, NAD-independent D-lactate dehydrogenase on lactate, and NADH oxidase on NADH (Anders et al., 1970; Daeschel, 1989; Dillon and Cook, 1994; Thomas and Pera, 1983). Hydrogen peroxide accumulates because these microorganisms do not produce the enzyme catalase, which degrades the compound into water and oxygen. The exact benefit of hydrogen peroxide to lactic acid bacteria is unknown. This is especially true insofar as it has been demonstrated that hydrogen peroxide is produced at autoinhibitory levels by some *Lactococcus* spp. (Anders et al., 1970; Gilliland and Speck, 1969). Gilliland and Speck (1969) showed that growth of *Lactobacillus* spp. was unaffected by hydrogen peroxide, despite the fact that these bacteria produced more hydrogen peroxide than the *Lactococcus* spp. Thomas and Pera (1983) suggested that hydrogen peroxide production by lactic acid bacteria may play a role in their microbial ecology. First, oxygen utilization in the production of hydrogen peroxide by lactic acid bacteria may benefit the group by giving them an edge over aerobic microorganisms. Second, release

of toxic metabolites such as superoxide, hydroxyl radicals, and hydrogen peroxide may inhibit growth and metabolism of other microorganisms.

A number of studies have been conducted to determine the contribution of hydrogen peroxide to the antimicrobial activity of lactic acid bacteria starter cultures under natural conditions. For example, Dahiya and Speck (1968) found that *Lactobacillus bulgaricus* and *L. lactis* inhibited the growth of *Staphylococcus aureus*. Inhibition was due partly to hydrogen peroxide. Storage at low temperature favored hydrogen peroxide formation, with the maximum at 5°C and pH 7.0. There was an inverse relation between acid production and hydrogen peroxide formation. Juffs and Babel (1975) evaluated commercial multistrain cultures containing *L. lactis* and *L. cremoris* added at 0.5% to milk containing various psychrotrophic microorganisms. Inhibition was demonstrated, but extent depended on temperature, time, starter type, and psychrotroph initial number and type. The antagonist was determined to be hydrogen peroxide, for catalase decreased inhibition. One culture produced a maximum of 0.000268% hydrogen peroxide after 72 hours at 7°C. Like Dahiya and Speck (1968), Juffs and Babel (1975) found that storage at low temperatures favored hydrogen peroxide formation, and an inverse relationship existed between acid production and hydrogen peroxide formation.

Gilliland and Speck (1977) demonstrated that hydrogen peroxide produced by *Lactobacillus acidophilus* was partly responsible for inhibition of *Staphylococcus aureus*, *Salmonella typhimurium*, and enteropathogenic *Escherichia coli*. Price and Lee (1970) studied the effect of added *Lactobacillus* on *Pseudomonas* spp. in oysters. They found that as *Lactobacillus* increased, *Pseudomonas* decreased, and they concluded that inhibition was due to hydrogen peroxide. In contrast to the previous studies, the research of Raccach and Baker (1978) found no significant production of hydrogen peroxide by the meat

starter culture bacteria *Pediococcus cerevisiae* or *L. plantarum* over the temperature range 10 to 35°C. Application of starter cultures to produce hydrogen peroxide is complicated by the fact that many foods contain some amount of catalase and superoxide dismutase.

As indicated, hydrogen peroxide is a reactant and a product in two enzymatic antimicrobial systems. In the lactoperoxidase system of raw milk (Banks et al., 1986; Reiter and Harnulv, 1984), the enzyme lactoperoxidase catalyzes oxidation of thiocyanate by hydrogen peroxide, producing antimicrobial hypothiocyanite ions, higher oxyacids, and short-lived oxidation products (Gaya et al., 1991). Potential sources of hydrogen peroxide for this reaction are the lactic acid bacteria. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide (Marth, 1966). White et al. (1963) reported that glucose oxidase, acting on glucose in honey, produced hydrogen peroxide as an inhibitory agent. Field et al. (1986) demonstrated that glucose oxidase could extend the shelf-life of refrigerated, fresh whole flounder, and flounder fillets stored at 2 to 4°C. Using glucose oxidase, Kantt and Torres (1993) inhibited the bacteria *Pseudomonas fluorescens* and *Acinetobacter calcoaceticus*, as well as the yeast *Hansenula polymorpha*, all of which are spoilage agents on shrimp. Their research demonstrated that hydrogen peroxide, and not pH reduction by gluconic acid, was responsible for inhibition of these microorganisms. Another microorganism, *Corynebacterium aquaticum*, was inhibited, however, by gluconic acid but not by hydrogen peroxide (Kantt and Torres, 1993). Hydrogen peroxide itself does not have antimicrobial activity. Rather, it produces powerful reaction products such as singlet or superoxide oxygen (O_2°), which is highly toxic to living organisms. Another possible mechanism of inhibition is through oxidation of sulfhydryl groups and double bonds in proteins and lipids (Cords and Dychdala, 1993).

4 Interactions of Antimicrobial Hurdles

Control of microorganisms, with the objective of achieving high-quality, safe food products characterized by adequate shelf-life for distribution and consumption, can be accomplished through manipulation of factors influencing the physiology of microorganisms. The factors include water activity (a_w), acidity, and chemical composition—including antimicrobials, atmosphere, and temperature. As intensity or level of each of these factors moves from the optimal level toward and beyond the minimal and maximal levels allowing microbial cell function, microbial growth rate decreases until it ceases completely, and death results.

Microbial control, i.e., inhibition or inactivation, can be achieved either by manipulating one factor so that it becomes bacteriostatic or bactericidal, or by using two or more factors (or hurdles) to achieve preservation (Leistner, 1985, 1995; Leistner and Rodel, 1976; Sofos, 1993). Examples of interactions of factors affecting microbial growth, e.g., pH, Eh, water activity, temperature, and chemicals, are abundant in the scientific literature and can be found throughout this report. As the intensity of one factor increases, the amount of the other needed for a given level of antimicrobial activity decreases; or a combination of multiple factors at low intensities achieve the same extent of antimicrobial activity as fewer factors applied at higher intensities do. These multihurdle interactions are well developed, not only in the scientific literature but also in many commercially available food products.

Throughout this report, there have been references to observations that the inhibitory activity of naturally occurring antimicrobials also is influenced by other parameters important in microbial physiology. Furthermore, the activity of naturally occurring antimicrobials can be enhanced when they are used in combination with other chemical agents or with physical parameters, such as heat or cold, that affect mi-

crobial growth. The concept of food preservation through multihurdle interactions should be exploited as efforts to develop new microbial preservation systems are undertaken. Combinations of natural antimicrobials with other inhibitory agents may allow food products to be preserved with low concentrations of compounds with intense flavoring or aromatic properties.

The search for multifactor food preservation systems can be facilitated through application of the concept of predictive microbiology (Buchanan et al., 1993; Farber, 1986; Labuza et al., 1992; McClure and Roberts, 1992; McMeekin et al., 1993; Ross and McMeekin, 1994). Through the use of experimental data and analysis with mathematical models, predictive microbiology allows the selection of hurdle combinations, including naturally occurring antimicrobials, to achieve the desired shelf-life. In addition, development and application of quantitative risk assessment models should allow a more accurate estimate of preservation needed in a given food system (Buchanan and Whiting, 1996; Whiting and Buchanan, 1994). Once a preservation system is designed and validated, its application in food production, processing, and preservation can be managed through the concept of hazard analysis critical control points (HACCP). Use of HACCP systems allows prevention of food safety problems through the monitoring of critical control points designed to control potential hazards (Council for Agricultural Science and Technology, 1994; International Commission on Microbiological Specifications for Foods, 1988; National Advisory Committee on Microbiology Criteria for Foods, 1990; Pierson and Corlett, 1992; Sofos, 1992; Tompkin, 1990). Application of the HACCP system is required through regulation in the processing of products such as low acid canned foods, meat, poultry, and seafood products.

5 Activity and Uses in Foods

Introduction

The activity of naturally occurring antimicrobial compounds may be enhanced or diminished when subjected to various processing conditions administered to foods. Elevated temperature and departure from pH and water activity conditions normally present in plant and animal tissues can result in changes in antimicrobial effectiveness. A factor limiting the use of many naturally occurring antimicrobials in processed foods is the undesirable flavor or aroma they may impart when present at highly effective concentrations. Many phenolic compounds responsible for the flavor of spices and herbs, for example, are antimicrobial. The use of spices and herbs for the purpose of controlling foodborne microorganisms is limited, however, by their undesirable sensory properties at high concentrations.

Traditional Antimicrobials

As indicated, the traditional antimicrobials, including sugars, salt, and wood smoke have been used extensively in food processing and preservation for thousands of years. In recent years, their importance and uses have diminished because of actual or perceived health concerns and associated consumer demands. The main function of the traditional antimicrobials in modern food products is as flavorings; their contribution to product preservation has been replaced to some extent by application of packaging treatments and widespread use of mechanical refrigeration. To obtain the necessary shelf-life and to ensure the safety of certain food products, however, additional hurdles are needed. Naturally occurring antimicrobials could be useful in fulfilling this function.

Organic Acid Antimicrobials

Some organic acid antimicrobials, e.g., acetic, lactic, benzoic, propionic, and sorbic acids, have found extensive use as general additives or antimicrobials in foods. Common applications of acetic acid and acetates include products such as mayonnaise, salad

dressings, mustards, pickles, olives, marinades, catsups, and other sauces. A combination of fumigation with acetic acid vapor and storage under modified atmosphere packaging increased the shelf-life of grapes and strawberries (Moyle et al., 1996). Acetic acid spray-washes during slaughter also are used to decrease microbial loads on animal carcass surfaces (Dickson and Anderson, 1992; Sofos, 1994b).

A patent (Berggren and Nocquet, 1995) has described a process for inhibiting pathogenic bacteria in chilled, cooked meats, which consists of a particulate encapsulated product containing acetic acid and an edible lipid. The process also is permitted for use as an acidifier in processed meat and poultry products. Sodium or calcium diacetate and calcium acetate inhibit molds and rope-forming bacteria in bread while having no adverse effects on baker's yeast (Doores, 1993). Use of acetic acid as a preservative in foods, however, is limited by its pungent odor and taste. An oxidized derivative of acetic acid is peracetic acid, which, in the presence of organic material, is oxidized to yield acetic acid and oxygen. Peracetic acid has been suggested as a disinfectant for food contact surfaces and equipment (Foegeding and Busta, 1991; Sofos and Busta, 1992).

Because of its low cost, among other advantages, benzoate probably is the most commonly used antimicrobial in commercial food preservation (Sofos and Busta, 1992). When there are concerns for flavor defects, benzoate may be used at lower levels in combination with other preservatives such as sorbate or parabens. In general, benzoates are used extensively in the preservation of foods with pH < 4.5. Use sometimes is limited because of the narrow pH range of activity, undesirable flavors, and a toxicological profile less desirable than that of other common antimicrobials (Chipley, 1993; Lück, 1980; Sofos, 1994a). Foods in which benzoate is used as a preservative include fruit products, beverages and bakery products, fruit juices and drinks (Figure 5.1), fruit salads and cocktails, salads and salad dressings, pickles, relishes, olives, sauerkraut, dried fruits and preserves, jams and jellies, and margarine. Other items preserved with benzoate are animal feeds, pharmaceutical prod-

ucts, and cosmetics. Amounts of benzoate used in the preservation of commercial products are in the range 0.05 to 0.1%.

Lactic acid, the dominant acid in fermented dairy products, inhibits most bacteria as pH decreases below 4.5; it is not effective against yeasts and molds (Doores, 1993). The compound also has been used in spray-washing of red meat and poultry carcasses to decrease bacterial contamination acquired during slaughter (Hardin et al., 1995; Smulders and Woolthuis, 1985). Sodium lactate also is an antimicrobial and flavoring agent used in processed meat and poultry products (Shelef, 1994).

The most common use of propionates is as mold and rope inhibitors in bread and other bakery products (Figure 5.2). In addition to their antimicrobial activity, propionates are useful in baked goods because they cost little and do not interfere with the yeasts involved in leavening. Use of calcium propionate is preferred in breads and rolls, where it contributes to enrichment with calcium. Because it helps avoid interference of calcium ions with chemical leavening, sodium propionate is preferred, however, for use in chemically leavened products such as cakes (Doores, 1993; Sofos, 1994a). In addition to inhibiting mold in cheese, propionates may be used as antimicrobials in processed cheese products, fruits, vegetables, jams, jellies, preserves, malt extracts, and tobaccos (Robach, 1980; Sofos, 1994a; Sofos and Busta, 1992).



Figure 5.1. Benzoate may be used as a preservative in many foods including fruit juices.

The most commonly used form of sorbates is the highly water soluble potassium salt. Common applications involve uses as antimicrobials in all types of human foods, animal feeds, pharmaceuticals, cosmetics, packaging materials, and other products. Foods preserved with sorbates include dairy products, bakery goods, fruit and vegetable products, edible fat emulsion products, certain meat and fish products, and confectionery items. It should be emphasized again, however, that antimicrobial agents, including sorbates, are neither substitutes for good sanitation nor agents to improve the quality of highly contaminated, partly spoiled, or otherwise degraded food products. Specific food products that may be preserved with sorbates include various types of cheeses; fresh, fermented, and pickled vegetables; dried fruits and juices; syrups; fruit cocktails; jams; jellies; preserves; beverages; wines; bakery goods; cakes; icings; toppings; fillings; dry sausages; smoked fish; margarine; mayonnaise; salad dressings; and delicatessen products (Sofos, 1989b).

Lipid Antimicrobials

Although monoacylglycerols and other lipid compounds have been used as emulsifiers in the food industry since 1932, limited attention has been paid to their antimicrobial potential in food systems. One of the earliest indications of the preservative activity of lipids was reported by Grecz et al. (1959), who presented evidence that fatty acids generated during ripening of natural cheese prevented *Clostridium botulinum* from producing neurotoxin. Japanese researchers seem to have carried out the most exten-



Figure 5.2. Propionates and sorbates are used as preservatives in foods such as bakery goods.

sive studies on the preservation of foods by means of lipids. Monoacylglycerols (C_8 to C_{10}) increased the shelf-life of various foods, including soy sauce, miso, wiener sausage, Worcestershire sauce, sponge cake, and noodles (Shibasaki, 1982). The relatively short-chain length suggests that activities were directed mainly towards fungi and yeasts. Monolaurylglycerol and fumaric acid prolonged shelf-life and prevented sliminess in *kamaboko*, i.e., a fish paste similar to *surimi* (Kabara, 1993). Monoacylglycerols have been used as antimicrobials for years in Japan, but their use has been limited by off-flavors and duration of action. Sugar esters with high heat stability were inhibitory at 0.005 to 0.03% to *Bacillus stearothermophilus*, *B. coagulans*, *Desulfotomaculum nigrificans* and various clostridia in canned milk, coffee drinks, and chicken soup, products that are popular in vending machines in Japan (Kabara, 1993). Monoacylglycerols also have been shown to enhance the activities of other antimicrobials such as nisin (Blackburn et al., 1989). These findings emphasize the importance of using mixtures, combinations, or systems—e.g., hurdles—of antimicrobials to increase their effectiveness in foods.

In the United States and in certain European countries, monolaurylglycerol, i.e., monolaurin, has been investigated quite thoroughly for its efficacy in food preservation (Kabara, 1993). Monolaurylglycerol, at 0.5%, was shown to prevent *Clostridium botulinum* types A, B, and E neurotoxin formation in meat products (Notermans and Dufrenne, 1981). Under the conditions tested, monolaurylglycerol was six times more effective than sorbic acid in delaying toxigenesis by *C. botulinum*. Monolaurylglycerol also showed potential for enhancing the safety and the shelf-life of various flesh foods, including deboned chicken meat, minced fish, and turkey frankfurter slurries (Baker et al., 1985; Hall and Maurer, 1986).

In dairy products and several other foods, fatty acids and monoacylglycerols were evaluated for antimicrobial activity against the Scott A strain of *Listeria monocytogenes* (Wang and Johnson, 1992, 1997; Wang et al., 1993). In whole milk and skim milk, certain fatty acids prolonged the lag phase, especially at 4°C. Monolaurylglycerol was bactericidal at 4°C in skim milk but was less inhibitory at 23°C and did not inhibit *L. monocytogenes* in whole milk. Mixtures of monoacylglycerols prepared by lipase-catalyzed glycerolysis and fractionation were effective in milk and in other foods (Wang and Johnson, 1992; Wang et al., 1993). The data suggested that CLA or monolaurylglycerol could be used as inhibitory agents against *L. monocytogenes* in certain dairy products or in other

foods containing decreased fat. Monolaurylglycerol and monocaprylglycerol also were found to inhibit *L. monocytogenes* in frankfurters, seafood salad, and Camembert cheese (Wang and Johnson, 1997). Unda et al. (1991) found sodium lactate and monolaurylglycerol to inhibit *Clostridium* and *Listeria* spp. in refrigerated beef roasts.

Monoacylglycerols and other polyhydric esters lowered the heat resistance of certain bacteria and fungi, a fact suggesting that these compounds could find use in lessening the heat-treatment requirements of some foods. The usefulness of monolaurylglycerol in heat inactivation of spores of *Bacillus stearothermophilus* was demonstrated by Kimsey et al. (1981). Lauric acid, monocaprylglycerol, and monolaurylglycerol enhanced thermal inactivation of *Escherichia coli* and *Pseudomonas aeruginosa* (Kato and Shibasaki, 1975). The mean D_{60C} -value for two *E. coli* O157:H7 strains in ground beef was significantly less in the presence of a combination of glycerol monolaurate, EDTA, and sodium lactate compared to control ground beef without antimicrobials (Greeson et al., 1996).

Many of the proposed applications of monoacylglycerols in foods have been directed towards control of yeasts and fungi. Monolaurylglycerol was useful in margarine for control of undesirable fungi (Kabara, 1993). Japanese researchers reported that monocaprylglycerol inhibited growth of a spoilage yeast in soy sauce (Shibasaki, 1982). Monocaprin inhibited a pellicle-forming yeast as well as *Aspergillus oryzae* and *Bacillus subtilis* (Kabara, 1993).

In conclusion, the antimicrobial activities of lipid compounds depend strongly on the substrate or food system preserved, on the presence of additional antimicrobials cooperating with or providing additional barriers to microbial growth, and on the presence of substances that can neutralize the antimicrobial activity of the lipid. Because fatty acids and other lipophilic compounds may be sequestered in the fatty phases of particular foods, the primary use of these compounds is in low-fat foods. The activities of monolaurylglycerol and certain other lipids are neutralized by starch or some other polysaccharides and by certain lipid binding proteins such as serum albumin. The necessity of using fatty acids and monoacylglycerols at high concentrations in certain foods also could have an adverse impact on the sensory characteristics of the food.

Antimicrobial Plant Substances

As has been indicated, numerous antimicrobial compounds known to be present naturally in plant

tissue have been studied primarily in the form of crude extracts. Minimal inhibitory concentrations against a large number of foodborne bacteria, yeasts, and molds have been reported in studies using pure cultures and laboratory media as a general protocol. Relatively few studies have described the inhibitory activities of naturally occurring plant substances against microorganisms actually suspended in foods. Observations on activities of those substances that have been studied in food systems do indicate, however, that higher concentrations are necessary to achieve levels of inhibition comparable to those noted in laboratory media.

Some foods are stored and preserved under olive oil in certain Mediterranean countries, and naturally present phenolic compounds, hydrogen peroxide, and the anaerobic environment are believed to inhibit microbial growth (Dallyn, 1994). The antimicrobial properties of mustard, clove, cinnamon, and their oils were, according to Boyle (1955), described in the late 1800s. Hoffman and Evans (1911) studied the inhibitory effects of these spices in apple juice and ketchup. Mustard suppressed microbial spoilage of mayonnaise based delicatessen salads.

Chung et al. (1990) evaluated the antimicrobial activity of extracts from Chinese medicinal plants added to cabbage juice. Growth of *Listeria monocytogenes* was suppressed by extracts from *tin men chu* and *sui mao heung*. This inhibition was prevented by protein but not by sodium chloride. The lethal effect of carrot juice on *L. monocytogenes* has been reported. Lethality and inhibition are greater in a pH range of 5.0 to 6.4 but are diminished in the presence of sodium chloride. Treatment with carrot juice of shredded lettuce inoculated with *L. monocytogenes* resulted in significantly smaller populations of the pathogen throughout a 14-day storage period at 5°C (Beuchat and Brackett, 1990; Beuchat et al., 1994; Nguyen-the and Lund, 1991, 1992).

Farbood et al. (1976) studied the bacteriostatic and bactericidal effects of rosemary spice extract in laboratory media and in mechanically deboned poultry meat, turkey breast, and beef. Growth inhibition of *Staphylococcus aureus* occurred in the laboratory media containing 0.1% extract. Inhibition was observed only at 5% in meats, however. The researchers concluded that the lipid in meat adsorbs the extract, thus decreasing its concentration in the aqueous phase and consequently its bactericidal effect.

Extracts of mace, bay leaf, and nutmeg added to turkey frankfurter slurries have been reported to inhibit the production of toxin by *Clostridium botulinum* (Hall and Maurer, 1986). Garlic and onion oils, at

0.15% in pork slurry, inhibited toxin production by *C. botulinum* type A. Toxin production by Types B and E was not inhibited (DeWit et al., 1979). Cloves, onion, ginger, and pepper at 1% concentrations in 10% meat slurry inhibited growth of *Staphylococcus aureus* (Nkanga and Uraih, 1981).

Shelef et al. (1980) studied the effects of sage and rosemary on growth of *Staphylococcus aureus*, *Salmonella typhimurium*, and *Bacillus cereus* in laboratory broth, boiled rice, and baby foods. The sage concentration required to inhibit growth of *S. typhimurium* in broth was three to ten times higher than that needed for *Staphylococcus aureus* and *B. cereus*. Growth inhibition by less than 2.5% sage in foods was limited to *B. cereus* and *S. aureus* in boiled rice and baby food. Fat and/or protein was more effective than carbohydrates were in protecting bacteria.

Natural antimicrobial compounds in some plant tissues have a broad spectrum of activity whereas extracts of other plant tissues exhibit activity against only gram-negative or gram-positive bacteria, or only against bacteria or fungi. Thus compounds responsible for inhibition evidently act against different cellular sites or metabolic functions. Little information is available on the mode of action of these compounds. Phenolic compounds are thought to interfere with the metabolic pathways associated with energy synthesis (Baranowski et al., 1980; Conner et al., 1984). The mode of action of the anticandidal component of garlic has been attributed to inactivating proteins, inhibiting sulfhydryl compounds, and disrupting enzyme function by oxidation of the binding site to -SH groups of allosteric sites (Barone and Tansey, 1977).

Mold growth was inhibited in raisin bread containing cinnamon (Bullerman et al., 1973; Hartung et al., 1973). Aflatoxin production in breads inoculated with *Aspergillus parasiticus* was studied by Bullerman (1974). Aflatoxin B₁ and G₁ were formed in rye, whole wheat, and white breads, but were at lower concentrations or absent in raisin bread containing 1% cinnamon.

Naturally extracted allyl isothiocyanate is permitted for use as a preservative in Japan (Isshiki et al., 1992). Approval of isothiocyanates is met with reluctance, however, because some compounds are thought to be goitrogenic, cytotoxic, or mutagenic. The existence of potential beneficial functions of isothiocyanates, such as inhibition of tumor formation, is a possibility, however (Delaquis and Mazza, 1995). Other concerns associated with the use of isothiocyanates or their precursors in food preservation are related to their intense, sometimes adverse, sensory characteristics, which are described as pungent or as very spicy.

These undesirable effects may be minimized if isothiocyanates are used in small amounts and in combination with other antimicrobial agents providing synergistic or additive activity.

Polypeptide Antimicrobials

Although polypeptides have potential for application in food preservation, the use of these substances by the food industry will depend on (1) potential for toxicity, including oncogenicity and antigenicity; (2) stability and activity in foods; and (3) inexpensive production on a large scale, or efficient production by starter organisms in fermented foods. These requirements currently are met by a limited number of polypeptides, including lysozyme, lactoperoxidase, glucose oxidase-catalase, and nisin. For several other peptides described, considerable research will be required, and potential use remains speculative.

Major advances have been made in the past 10 years in scientists' understanding of the mode of action of natural polypeptides inhibiting or inactivating bacteria and fungi. Polypeptides inhibit by three general mechanisms: (1) lysis (digestion) of cell wall proteins and polysaccharides, (2) insertion into membrane with disruption of membrane function, and (3) formation of toxic molecules such as oxygen radicals, which react with essential cell constituents. The various polypeptides often work in concert and are active only in specific food systems and tissues. Several systems inhibit or kill bacteria and fungi nonspecifically and could not be used when microbial activities are essential, as in the production of certain fermented foods. Nonspecific systems such as the lactoperoxidase system, however, could be used to decontaminate raw foods such as poultry or fish. Other polypeptides such as lysozyme, bacteriocins, and lactoferrin selectively inhibit limited groups of organisms, and this selectivity is of great utility in fermenting foods such as cheese and wines.

Biopreservation of foods by enzymes could limit thermal denaturation of nutrients and the requirements for chemical preservatives. Enzymatic preservation has advantages over chemical preservatives, including low temperature incubation requirements and minimal interference by side reactions, i.e., specificity (Pecher et al., 1990). Furthermore, activity is controlled readily by external environmental parameters such as temperature. Overall, the potential for use of polypeptides is in large part unrealized, and studies in foods are necessary to evaluate the efficacy of this promising group of molecules.

Currently, the bactericidal substance egg white

lysozyme has found limited application as an antimicrobial in the food industry (Table 5.1). This substance is used primarily to prevent gas formation or "late blowing" by *Clostridium tyrobutyricum* and other gas-producing *Clostridium* spp. during ripening of certain cheeses such as provolone, Grana, Padano, Emmental, and Gouda (Carini et al., 1985). Lysozyme usually is added to the milk vat at 0.002 to 0.0035%, and it associates nearly completely within the curd, yielding levels in the cheese of 0.02 to 0.04%. When used at this level, it generally does not inhibit the activities of starter organisms although some inhibition of natural whey cultures occurred in Grana cheese production, probably because of its activity against *Lactobacillus helveticus* (Neviani et al., 1992). Starter organisms can be developed that are resistant to lysozyme, however. The substance is approved, or approval is pending, for use in cheeses in several countries (e.g., Austria, Australia, Belgium, Canada, Denmark, Finland, France, Italy, Norway, Spain, the United Kingdom, and the United States).

Hen egg white lysozyme has been evaluated as a preservative in various foods in addition to cheese and as a preservative of pharmacological products (Hayashi et al., 1989; Procter and Cunningham, 1989). Japan and Korea have investigated lysozyme use to a much greater extent than most Western and European countries have. Japanese investigators have investigated lysozyme as a preservative in foods such as fresh vegetables, tofu, sausage, fish cakes, and seafood (Tranter, 1994). Applications include coating

Table 5.1. Types of foods in which lysozyme has been used as a preservative (from Tranter, 1994).

Food system	Preservative
Vegetables	Lysozyme
Fish	Lysozyme
Meat	Lysozyme
Fruit	Lysozyme
Tofu bean curd	Lysozyme
Sausages	Lysozyme
Bacon	Lysozyme
Cheese	Lysozyme
Butter	Lysozyme
Seafood	Lysozyme and NaCl
Fish cakes	Lysozyme and NaCl
Wine	Lysozyme and β hydroxybenzoic esters
Sake	Lysozyme and β glycanpyranose
Potato salad	Lysozyme and amino acids
Sushi	Lysozyme and vinegar
Chinese noodles	Lysozyme, amino acids, and polypropylene glycol
Creamed custard	Lysozyme, amino acids, and ethanol
Infant milk formula	Lysozyme, lactoferrin, and antibodies

fresh fruit, vegetables, meat, and fish surfaces, as well as adding to wine and *saké*. Lysozyme extends the storage stability of *saké* and *mirin*, Japanese wines, and prevents malolactic fermentation in wines produced in Europe and the United States (Dell'Acqua, 1993; Pitotti et al., 1991).

As a specific example of the use of lysozyme in seafoods, the shelf-life of *kamaboko* was extended by dipping the product in a 1% gelatin/0.05% lysozyme mixture (Akashi and Oono, 1972). The treated food had fewer viable bacterial counts and less sliminess and brown color development than the untreated product during storage at 10°C for 14 days. Bacteria isolated from the slimy *kamaboko* (not treated with lysozyme) included *Pseudomonas* (22%), *Leuconostoc* (19%), *Achromobacter* (19%), *Micrococcus* (12%), lactic streptococci (8%), yeast (5.2%), and *Bacillus* (4.2%). Because the isolated gram-negative bacteria contributed to slime formation, lysozyme may have been active against this group of bacteria. Addition of lysozyme to infant formula milk supported growth of *Bifidobacterium bifidus* in the intestines, as does human milk (Sawada et al., 1967).

The lactoperoxidase/thiocyanate/hydrogen peroxide system has been utilized industrially and has been suggested as a preservative in several systems, including the intermediate preservation of raw milk in developing countries where refrigeration is unavailable (Ekstrand, 1994; International Dairy Federation, 1988; Ridley and Shalo, 1990). Freshly treated bovine milk can attain 22 hours of additional storage life at 15 to 20°C, and much longer periods if the milk is kept below 5°C and hydrogen peroxide and thiocyanate are in adequate supply (Ridley and Shalo, 1990). The oxidative intermediates are unstable and shortlived. Thiocyanate is stable and can be added once, but it is necessary to continuously add or to generate hydrogen peroxide, which is unstable. In addition to preventing milk spoilage, the lactoperoxidase system can help kill or prevent growth of milkborne bacterial pathogens. The lactoperoxidase system has been found to inactivate *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *E. coli* O157:H7, and *Yersinia enterocolitica* in milk and in infant milk formula (Earnshaw et al., 1990; Ekstrand, 1994; Farrag and Marth, 1992; Reiter and Harnulv, 1984).

Lactoperoxidase has potential for preservation of various foods in addition to milk and cheeses. It has been used to prevent spoilage of infant milk formula, soft ice cream, and pastry cream (Johnson, 1994). The activity of the lactoperoxidase system against gram-negative pathogens suggests that it could be used to preserve other foods, particularly those that are of

animal origin and may contain enteric pathogens. There are unpublished reports of the use of lactoperoxidase system in toothpastes, infant formulas, and bottled salad dressings, but little or no research has been done to determine the effectiveness of lactoperoxidase system treatment of meat, poultry, or fish products. The potential for lactoperoxidase system use in meats is suggested by observations of a similar system based on myeloperoxidase (a muscle peroxidase), which is known to be strongly antibacterial in human tissue. In contrast to certain other polypeptide antimicrobials, the lactoperoxidase system is nonspecific and could not be targeted to inactivate individual species within a large population in a given food; hence, it could be quite effective in controlling bacterial levels on raw meats such as poultry, in which microbial flora does not promote product stability.

An interesting potential application of lactoperoxidase is as a probiotic (Reiter et al., 1981). Calves fed milk supplemented with the lactoperoxidase system components showed better weight gain than calves receiving unsupplemented milk did. The lactoperoxidase system seems to offer a healthful means of preventing pathogenic growth and consequences. A novel application of the lactoperoxidase system could be its use in combination with glucose oxidase and monoclonal antibodies in tumor therapy (Ekstrand, 1994).

Glucose oxidase is used in foods for microbial control and removal of oxygen. It is used in egg albumin, egg yolk, whole egg, dried meat, and potatoes to prevent browning and formation of off-flavors (Low et al., 1989; Pitcher, 1980). Coupling of glucose oxidase with catalase provides an effective method of enzymatically deaerating various foods. The combination of enzymes decreases dissolved oxygen in orange juice, prevents browning, and retards growth of aerobic yeasts (Sagi and Mannheim, 1990). The same principle is used to decrease growth of wild yeasts in draft beer (Blockmans et al., 1987) and wine (Ough, 1975). The enzyme system has been used in oxygen removal from the headspace of packages and as a stabilizing agent in citrus soft drinks, cola beverages, beer, wine, syrups, mayonnaise, and salad dressing. Glucose oxidase treatment also has been reported to prolong the shelf-life of freshly caught seafood (Field et al., 1986; Shaw et al., 1986; Wesley, 1982).

A limited number of studies have examined the antibacterial effect of conalbumin and lactoferrin in foods. Some reports have found that these substances inhibit certain organisms, e.g., *Listeria monocytogenes*, but only when used at high concentrations (approximately 0.03% apolactoferrin) and in specific foods (Payne et al., 1990). It is unclear why conalbumin

min is highly active in egg white but not in other foods. It is likely that other factors in egg albumen promote the antimicrobial activity of conalbumin. These iron-chelating proteins may find increasing uses in a limited number of foods, probably in combination with other factors to help restrict the availability of iron. The use of conalbumin and lactoferrin in foods may depend, however, on economics of scale and on the production of the protein. Biologically active human lactoferrin was cloned and expressed by *Aspergillus oryzae* at levels as high as 0.0025% (Ward et al., 1992). This system should provide an economical source of pure lactoferrin for research and for possible application in foods.

Lactoferricin and related peptides derived from food grade proteins would seem to have considerable potential as antimicrobials in foods. But studies of effectiveness in food systems are necessary to evaluate properly the potential of lactoferricin as a food preservative.

As antimicrobial agents in foods, bacteriocins can be considered one of several hurdles or barriers (Muriana, 1996). The contribution of bacteriocins to a complex, multifactor food preservation system could be as bactericidal agents decreasing levels of contamination and thus enhancing inhibition by other hurdles.

Nisin is classified as GRAS for use in a limited variety of food products (Ronk, 1988). Use of nisin is permitted in approximately 50 countries, among which are the United States and the United Kingdom, but the lists of foods to which it may be added differ. One approved use is its application in processed cheeses and spreads for inhibition of spoilage, e.g., "blowing," or toxigenic sporeforming bacteria such as *Clostridium sporogenes*, *C. butyricum*, *C. tyrobutyricum*, *C. botulinum*, and *Bacillus* spp. (Delves-Broughton, 1990; Hoover, 1992; Somers and Taylor, 1987; Thomas, 1977). Use of nisin-producing bacteria in cheddar cheese manufacture, and inclusion of such nisin-containing cheese in the production of pasteurized process cheese or cold pack cheese spreads could help control undesirable microorganisms (Zottola et al., 1994). The nisin-based, commercially available antimicrobial preparation, Nisaplin® inhibited 27 of 30 bacterial isolates originating from salad dressings; thus, nisin may help inhibit spoilage and extend the refrigerated shelf-life of salad dressings (Muriana and Kanach, 1995).

In addition to being permitted for use in processed cheese, nisin is permitted for use in milk in certain countries in the Middle East in order to enhance shelf-life at higher temperatures and long-distance distribution under inadequate refrigeration. Levels need-

ed for inhibition range from 100 to > 500 IU/mL and depend on pH, sodium chloride, phosphates, other preservatives, and other legal requirements (Hoover, 1992). For use in dairy products, nisin may be available as a component of skim milk powder. Inclusion of nisin in powdered milk can help make the product available in parts of the world with limited resources and unsafe water supplies (Hoover, 1992). Addition of nisin to cottage cheese or ice cream increased the rate of inactivation of *L. monocytogenes* (Dean and Zottola, 1996; Ferreira and Lund, 1996).

Other products showing increased shelf-life when exposed to nisin include liquid eggs, canned vegetables, fruit brandies, meats, fish, alcoholic beverages, and bakery products such as crumpets, and flapjacks (Delves-Broughton and Gasson, 1994). In liquid eggs, nisin has extended the shelf-life and inhibited the sporeforming pathogen *Bacillus cereus* (Delves-Broughton et al., 1992). The activity of nisin alone or in combination with lactate, citrate, EDTA versus sodium hexametaphosphate, against *Salmonella typhimurium* and *Escherichia coli* O157:H7 in raw beef or poultry meat was less than that in water or buffer (Cutter and Siragusa, 1995a, b; Mahadeo and Tatini, 1994). Shefet and colleagues (1995) found, however, that nisin combinations with food grade chelating agents decreased viable *S. typhimurium* counts on chicken meat by > 3 logs after exposure for 30 min. Immobilization of nisin in 1% calcium alginate gel decreased number of *Listeria monocytogenes* cells on pork; such treatments, therefore, may have potential use in decontaminating meat products (Fang and Cyi-Chenn, 1995). Nisin decreased numbers of *Listeria innocua* and *Brochothrix thermosphacta* in beef refrigerated under vacuum (Cutter and Siragusa, 1996a, b). Spray-washing of beef carcasses with nisin decreased populations of gram-positive bacteria (Nettles and Siragusa, 1994).

Use of nisin in low-acid canned foods can control thermophilic spoilage bacteria that may survive the canning process and grow under prolonged storage in warm environments. In addition, nisin can control *Clostridium pasteurianum* and *Bacillus macerans* spoilage in canned tomatoes and other high-acid foods. Nisin production *in situ* or addition of purified nisin can be used to control cabbage fermentation (Breidt et al., 1995). In high-moisture flour batters, nisin can control *B. cereus*, which survives mild heating.

The antimicrobial activity of nisin in muscle food product formulations is inconsistent due to potential interference by the phospholipids binding nisin to the meat surface and causing poor solubility, uneven distribution, and enzymatic degradation. In fish stored

under modified atmospheric conditions inhibiting spoilage, nisin may be useful in inhibiting toxin production by psychrotrophic *Clostridium botulinum* type E spores, which can grow under these conditions. Furthermore, nisin may be useful in beer and wine as an inhibitor of lactic acid bacteria (Delves-Broughton and Gasson, 1994). In Eastern Europe, nisin has been used to decrease the heat treatment of canned vegetables, thus saving energy and improving product texture, appearance, and nutritional quality (Hoover, 1992). Nisin also may be useful in alcoholic beverages, in which it may inactivate lactic acid bacteria and allow reduction in the amount of sulfites, which can cause allergic reactions, that are used for that purpose (Hoover, 1992).

With the exception of nisin, no bacteriocin has been approved for use in foods even though many bacteriocins are produced as by-products during growth of GRAS lactic acid starter culture bacteria (Muriana, 1996). Use of purified bacteriocins as food additives or as GRAS substances, would require FDA approval, as was the case for nisin (Muriana, 1996; Ronk, 1988). Legal use also can be allowed if a company chooses to self-affirm a bacteriocin as GRAS, as provided in the Code of Federal Regulations (Code of Federal Regulations, 1992).

The potential application of bacteriocins as natural food antimicrobials, after FDA approval, could be as purified bacteriocin preparations, as natural bacterial cell preparations of cultures producing bacteriocins, or through genetic expression of bacteriocin production in other food-grade microorganisms. Use of bacteriocins in meat and plant products may require, in addition to FDA approval, U.S. Food Safety and Inspection Service (FSIS) and U.S. Environmental Protection Agency approval, respectively, whereas genetic expression of bacteriocins in food-producing domestic animals may fall under the animal drug regulations (Owen Fields, 1996).

Overall, bacteriocins seem to have the greatest potential for preservation of raw and minimally processed refrigerated foods when a number of barriers can be used to prevent pathogenic growth and bacterial spoilage. Like other antimicrobials, bacteriocins have drawbacks and limitations when used in food. Most are inhibitory against only closely related organisms and are inactive against gram-negative bacteria and fungi. Target organisms differ in sensitivity and may gain resistance to bacteriocins by loss of a specific receptor. Bacteriocins are active over a limited pH range, are hydrophobic, may not be distributed evenly throughout a food system, and are inactivated by proteolytic enzymes and other substances in

foods. Unless technological advances can overcome these drawbacks, the effectiveness of bacteriocins may be limited to relatively homogenous foods with a favorable environment, e.g., pH or fat content. Although extensive research on bacteriocins has been conducted in the 1980s and the 1990s, additional evaluations are needed, and the potential in food systems needs to be exploited. Furthermore, there is a need for the application of sensitive bacteriocin detection methods to foods, for regulatory purposes (McMullen and Stiles, 1996).

The actual roles of and the potential for biological control by killer yeasts in food fermentations deserve further study. Defensin peptides should be tested for their toxicological properties and their efficacy as antimicrobials in food systems. Use of antibacterial peptides such as cecropins will require an improved understanding of their mechanisms of action and the design of peptides with activity specific for undesirable microorganisms. Specificity of antimicrobial activity, as well as economics of isolation and stability in foods, could determine usefulness. The cationic peptides with bacterial membrane penetrating activity, such as attacins, apidaecin, and abaecin deserve to be tested as antimicrobials in foods.

In addition to the compounds discussed, biological organisms have evolved a repertoire of antibacterial polypeptides to guard against infection by bacteria, fungi, and viruses. Many of these proteins have yet to be discovered and characterized. Some could have utility in food preservation. It might be useful to examine the evolutionary origin of antimicrobial polypeptides and to look for novel antibacterial peptides in organisms such as yeasts and filamentous fungi, which must thrive in mixed cultures and in fermented foods. Studies of these organisms might lead to the discovery of novel polypeptides with applications in food preservation.

Miscellaneous Microbial Metabolites with Antimicrobial Activity

Many products, including cheeses, fermented meats, cereals, legumes, and alcoholic beverages (Table 5.2), are preserved through the action of naturally present microorganisms or added microbial starter cultures, but the mechanism of inhibition may be due to more than one factor, including the antimicrobial substances produced by useful cultures (Dillon and Cook, 1994). In addition to organic acids, which lower pH, and to bacteriocins, which have a narrow

spectrum of activity, other metabolites in product preservation should not be ignored. One potential concern in the use of microorganisms as biopreservatives is the ability of certain strains under certain conditions to form toxic biogenic amines through decarboxylation of amino acids (McCabe, 1986; McMullen and Stiles, 1996).

Only recently has ethanol been evaluated as a potential antimicrobial in foods. Most studies have considered the antimycotic activity of the compound. Increased shelf-life of bakery products such as pizza crust was obtained by addition of ethanol and sterile water as a spray or a dip (Seiler and Russell, 1991). Other studies, by Seiler (1978), of breads, pizza crusts, rolls, cakes, and unbaked pastries demonstrated that ethanol sprayed to give final concentrations of 0.25 to 1.0% (w/w) was an extremely effective mold inhibitor. Seiler showed that 0.75% (w/w) of ethanol in bread was sufficient to double the shelf-life. This research first demonstrated that ethanol is a vapor phase inhibitor (Seiler and Russell, 1991); thus, techniques such as vacuum packaging with ethanol, adding sachets or strips impregnated with ethanol, or encapsulating are possible methods for incorporating ethanol as a mold inhibitor. Smith et al. (1987) found that

ethanol in gas-packaged apple turnovers inhibited yeast growth for 21 days at room temperature whereas turnovers packaged with CO₂/N₂ spoiled in 14 days. A decreased growth rate of rope-forming bacteria (*Bacillus subtilis*) in rolls was achieved at 1.0% (w/w) ethanol (Seiler and Russell, 1991).

All application studies with natamycin have involved use of the purified compound. Nilson et al. (1975) determined the effect of natamycin (0.01%) on the shelf-life of cottage cheese stored at 4.4, 10.0, or 15.6°C. Natamycin added in the wash water increased the time to spoilage of uninoculated cottage cheese by 13.6, 7.7, and 6.3 days over the control when stored at 4.4, 10.0, and 15.6°C, respectively. Cottage cheese inoculated with *Aspergillus niger* stored at the same temperatures had increased time to spoilage of 12.7, 6.0, and 4.3; samples inoculated with *Saccharomyces 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, 10.0, and 15.6°C, the inoculated and uninoculated cottage cheese had increased respective days to spoilage ranges of 20.4 to 26.7, 9.7 to 12.3, and 2.6 to 5.0.

Table 5.2. Examples of microorganisms which have been used in preservation of foods or feeds around the world (from Dillon and Cook, 1994)

Organism(s) added	Food/feed system	Target organism(s)
Bacteriophage	Beef	<i>Pseudomonas</i> spp.
Lactic acid bacteria	Milk	<i>Pseudomonas putida</i>
Lactic acid bacteria	Milk products	Pathogenic bacteria
Lactic acid bacteria	Poultry	<i>Clostridium botulinum</i>
Lactobacilli	Beef	<i>Brochothrix thermosphacta</i>
Streptococci	Milk	<i>Salmonella typhimurium</i>
<i>Lactobacillus delbrueckii</i> subsp. ^a <i>bulgaricus</i>	Milk	<i>Pseudomonas fragi</i>
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	Milk	<i>Listeria monocytogenes</i>
<i>Lactobacillus casei</i> subsp. <i>casei</i>	Shrimp	<i>Vibrio parahaemolyticus</i>
<i>Lactobacillus plantarum</i>	Bacon	<i>Clostridium botulinum</i>
<i>Lactobacillus plantarum</i>	Cheese	<i>Staphylococcus aureus</i>
<i>Lactobacillus plantarum</i>	Cheese	<i>Staphylococcus aureus</i> ; <i>Salmonella typhimurium</i>
<i>Lactobacillus plantarum</i>	Tempe	<i>Staphylococcus aureus</i>
<i>Lactobacillus reuteri</i>	Herring fillets	Spoilage bacteria
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	Cheese	<i>Staphylococcus aureus</i>
<i>Lactococcus lactis</i> subsp. <i>lactis</i> serovar <i>diacetylactici</i> , <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i>	Cottage cheese	Gram-negative psychrotrophic bacteria
<i>Pediococcus acidilactici</i> , <i>Lactobacillus plantarum</i>	Sausages	<i>Staphylococcus aureus</i>
<i>Pediococcus acidilactici</i> , <i>Lactobacillus plantarum</i>	Poultry	Psychrotrophic bacteria
<i>Eurotium chevalieri</i>	Rice	<i>Aspergillus parasiticus</i> (toxigenic)
<i>Aspergillus flavus</i> (nontoxigenic)	Maize	<i>Aspergillus flavus</i> (toxigenic)
<i>Candida guilliermondii</i>	Apples, citrus fruits	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp.

^aSubspecies

Lück and Cheeseman (1978) demonstrated that 0.05 or 0.1% of natamycin delayed mold growth on cheese for up to 6 months but did not prevent it completely. Verma et al. (1988) compared the antimicrobial effectiveness of natamycin with that of sorbic acid, benzoic acid, and nystatin in inoculated and uninoculated butter and cheese. All four preservatives decreased fungal growth on uninoculated butter and cheese samples stored 30 days at 7°C more than on the controls. In inoculated samples, nystatin was the most effective antifungal agent, followed by natamycin. Lodi et al. (1989) found that natamycin preserved Italian cheeses and had no detrimental effect on ripening.

Early work with natamycin suggested it might inhibit fungal growth on fruits and meats as well as in dairy products. Ayres and Denisen (1958) investigated several antifungal agents, including natamycin, for their potentials to extend the shelf-life of berries. Strawberries, raspberries, and cranberries were dipped in solutions containing up to 0.01% of each of the antibiotics and stored at $5 \pm 3^\circ\text{C}$ for various periods. Natamycin was one of the most effective of the antifungal agents tested. At 0.001 to 0.005%, it decreased fungal growth on strawberries, and 0.005% maintained fungal counts equal to or smaller than initial count for 9 days. Natamycin also prolonged the shelf-life of raspberries and cranberries. When sprayed on raspberries and strawberries in the field, a 0.005% concentration was more effective than the control in lowering percentage of fruit deterioration during subsequent storage.

Shirk and Clark (1963) investigated the effectiveness of natamycin against yeast spoilage of orange juice. At 0.002%, natamycin immediately decreased viable yeasts in uninoculated and inoculated (*Saccharomyces cerevisiae*) samples and eliminated viable yeast cells within one week of storage at 2.5 to 4°C. No spoilage was detected in inoculated or uninoculated natamycin-treated samples after 8 weeks of storage whereas, after one week of storage, inoculated control samples were spoiled. In a second study, natamycin at 0.0005% eliminated viable yeast cells in orange juice inoculated with natural contaminants and stored for as many as 12 weeks at 2.5 to 4°C. The uninoculated control sample spoiled in approximately four weeks, and the inoculated control in one week. The authors concluded that natamycin was of potential use in orange juice because it was an effective antimycotic and caused no off-flavors.

Ayres et al. (1956), evaluating several antibiotics including natamycin against spoilage fungi of raw, cut-up chicken, added antibiotics at 0.001% to chill-

ing water (1.7°C) and dipped chicken for 2 hours. Rimocidin and natamycin, alone and in combination with chlortetracycline (all at 0.001%) were the most effective antifungal agents and inhibited yeast growth on chicken stored 12 to 15 days at 4.4°C. Natamycin at 0.01% was effective against five molds, including *Aspergillus flavus*, isolated from bakery products. The compound prevented growth of molds and yeasts in quarg fillings and icings at 0.05% and in cream fillings at 0.01% (Ticha, 1975).

Natamycin is approved for application as a surface treatment of cheeses in approximately 30 countries (Shibata et al., 1991). In the United States, natamycin was approved for use in cheese making as a mold spoilage inhibitor in 1982. An aqueous solution containing 0.02 to 0.03% of natamycin may be sprayed on the surface of cuts and slices of cheese. Natamycin has an advantage over sorbates for use in cheese because it does not migrate readily into the cheese and does not affect flavor or appearance adversely (Andres, 1982; De Ruig and Van den Berg, 1978). Regulations in The Netherlands limit natamycin concentrations on cheese surfaces to ≤ 2 mg per dm² (surface) and ≤ 1 mm in depth (Daamen and Van den Berg, 1985).

Hydrogen peroxide has been proven effective as a preservative in milk (Eapen et al., 1975; Winger, 1951), half-and-half cream (Collins and Dirar, 1969), swiss cheese and cheddar cheese (Roundy, 1958; Wasserman, 1959), and cultured buttermilk and sour cream (Pack et al. 1968). Lillard and Thomson (1983) showed, in contrast, that only high concentrations of hydrogen peroxide, i.e., 0.53 to 1.2%, were able to inactivate microflora in poultry chill water and on poultry carcasses. At those levels, catalase from poultry blood reacted with the hydrogen peroxide and caused commercially undesirable bleached and bloated carcasses.

In the United States, hydrogen peroxide is allowed as a direct additive at 0.05% to pasteurize milk for making certain types of cheese and as an antimicrobial in whey (0.04%) and starch (0.15%) (Code of Federal Regulations, 1992). Catalase is added to products to remove residual hydrogen peroxide. It also is allowed for sterilization of polymeric food packaging surfaces, which generally are used for aseptically packaged foods and for sanitizing of food contact surfaces (Code of Federal Regulations, 1992). Hydrogen peroxide has been used to produce low calorie flour from waste products, industry wheat, straw, corn stalks and cobs, oat, rice, soybean hulls, spent brewers grain, and almond and peanut skins (McNeillie and Bieser, 1993).

6 Potential for Applications

To apply a naturally occurring antimicrobial to a food requires that one determines the efficacy of the compound both in vitro, i.e., in microbiological media, and in food products. The in vitro testing gives an indication of the antimicrobial effectiveness of a compound, and a number of variables or factors concerning the antimicrobial can be evaluated. One of these factors is the microorganism itself. As stated, the antimicrobial spectrum of a compound should be as broad as possible. As has been shown throughout this report, however, antimicrobial activity often depends on the type, genus, species, or strain of microorganism tested. For example, bacterial spores are much more resistant to the effects of antimicrobials than vegetative cells are. The cell wall type, i.e., gram-positive versus gram-negative, also has an important influence on the effectiveness of a number of compounds. Lipophilic compounds generally are less effective against gram-negative than against gram-positive bacteria. This is due to the presence of an outer membrane consisting of lipopolysaccharides, proteins, and lipids and acting as a barrier to the entrance of chemical agents (Russell, 1991). Damage to this barrier by chemicals such as EDTA or by physical agents such as freezing leads to the sensitivity of gram-negative bacteria to certain antimicrobials such as lysozyme or nisin.

Another factor that could influence resistance of a microorganism to an antimicrobial is sublethal injury caused by exposure to heating or freezing, for example. According to Board and Gould (1991), injury, which translates to increased susceptibility, should be exploited in developing a microbial control system involving antimicrobials. One major problem that has been demonstrated is that exposure of a cell to one stress may elicit the production of protection factors against subsequent stresses. *Salmonella* strains grown in acidic conditions (pH 5.2 to 5.8) have been shown to increase their resistance to β -lactam antibiotics, heat, salt, and lactoperoxidase systems (Laub et al., 1989; Leyer and Johnson, 1993).

Another important variable associated with successful use of an antimicrobial in a food is the initial number of microorganisms in the system. Because

most antimicrobials are bacteriostatic rather than bactericidal, the greater the initial number, the shorter the shelf life of the product. 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.

Many important intrinsic and extrinsic factors or variables associated with application of an antimicrobial to a food could be determined during in vitro testing (Parish and Davidson, 1993). They include temperature, atmosphere, pH, oxidation-reduction potential, and water activity. These tests will demonstrate potential problems that may be encountered in food application testing. Finally, it may be useful to evaluate the effect of certain food system components, such as lipids, proteins, and divalent cations, that might influence the effectiveness of antimicrobials (Rico-Munoz and Davidson, 1983, 1984). Lipids may decrease activity of lipophilic compounds, and, since many food antimicrobials are hydrophobic in character, there invariably will be some reduction. Proteins may cause binding of some compounds and reduce activity. Divalent cations may affect the activity of some compounds by affecting the microorganism itself or by interacting with the antimicrobial.

If a compound is to be useful as a natural food antimicrobial, however, it must function in a food system. A number of natural antimicrobials exist that could be used immediately in foods, but few actually have been utilized—mainly because their effectiveness in foods has not been investigated thoroughly (Dillon and Board, 1994). Application testing can be very complex and include a number of variables (Parish and Davidson, 1993). Most variables already have been mentioned or discussed and can be classified as microbial, food related (intrinsic), environmental (extrinsic), or process related (Gould, 1989). Because of the variation in characteristics and activities among naturally occurring compounds, it is somewhat difficult to generalize regarding methods for applying the compounds. Even among compounds such as benzoic acid or sorbic acid, which are classified as “synthetic” antimicrobial preservatives and approved by U.S. reg-

ulatory agencies, there are no standard methods for evaluating activity or application procedures. Selection of application procedures of the antimicrobial to a food involves use of either a model food system or the actual food. A great deal of information can be gained by using model systems containing a percentage, e.g., 10%, of a food in a buffer or microbiological medium. These systems demonstrate potential interferences by food components but allow for simplified sampling by the researcher. The microorganism or microorganisms utilized should be natural contaminants or pathogens of interest, and incubation conditions should reflect use and abuse. According to Dillon and Board (1994), in natural ecosystems, many antimicrobials act together and therefore might be most appropriately evaluated in combination or in combination with decreased levels of approved antimicrobials. Success of application testing may be determined by increased shelf life or decreased potential health hazards.

The best method for determining the antimicrobial type to be used may be based on understanding their mechanisms of action and/or their targets in the cell. However, the exact mechanisms through which antimicrobials affect microbial growth are complex and difficult to elucidate (Russell, 1991). 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 (Branen, 1993). Few targets, even for approved food antimicrobials, such as organic acids, actually have been elucidated fully. If the mechanism of the compound is known, combinations of antimicrobials with different mechanisms could be utilized against the microorganisms in the food product.

Properties of the antimicrobial compound must be addressed in formulation of an application scheme. For example, the purity of the compound would need to be known for the purpose of specifications and likely for regulatory approval. Related is the need for development of an assay method for a compound to determine input levels and stability. Compound polarity influences both solubility in a food and antimicrobial activity. Some of the best chemical antimicrobials are both hydrophobic and hydrophilic in character (Branen, 1993). Hydrophilic character allows for water solubility, which is the domain of the microorganism. Hydrophobicity allows the antimicrobial to react or adsorb with the cell membrane of the microorganism. At the same time, hydrophobic character increases, lipid solubility in food increases, which will limit

the exposure of microorganisms residing in the water phase. The pKa, or dissociation constant, of a compound has been mentioned numerous times in this report as a major factor in the activity of potential antimicrobials. The pH and buffering capacity of a food product to which a compound will be applied therefore could affect activity significantly. The chemical reactivity of an antimicrobial with other compounds of a food system is important. Chemical reactions can lead to decreased activity, in addition to altered sensory properties of a food (Branen, 1993). For example, some compounds are highly susceptible to oxidation or hydrolysis. Antimicrobial peptides, such as bacteriocins, are susceptible to loss of activity through proteolysis, binding, and destabilization by metals, agitation, freeze/thaw, or shearing (Daeschel, 1993). Enzymes are susceptible to some of the same factors, in addition to loss of activity due to pH, salts, and temperature (Fuglsang et al., 1995).

Perhaps the most important aspect of any compound proposed for use as a food preservative would be its toxicological aspects. Because they occur in nature, naturally occurring antimicrobials often are thought to be less toxic than synthetic compounds. Obviously, this is not always true. A naturally occurring antimicrobial must be demonstrated to be non-toxic either by animal testing or by its continuous consumption by consumers as a food component over a long period. The latter may be problematic even for some common potential natural antimicrobials such as spice extracts. For even though spices have been consumed for centuries, they normally are not consumed in the concentrations necessary to achieve antimicrobial activity. In addition to lacking toxicity, naturally occurring compounds must be nonallergenic (Harlander, 1993) and be able to be metabolized and excreted so as to not lead to residue build-up (Branen, 1993). Food antimicrobials should not react either to make important nutrients unavailable to humans or to destroy these nutrients.

As indicated, food additives in general and preservatives in particular are regulated in the United States by agencies dealing with food products, e.g., the FDA and the USDA-FSIS. One of the supposed attractions of naturally occurring antimicrobials is their decreased impact on the labeling of food. Consumers reportedly are concerned about the presence of synthetic chemicals in their foods and would prefer natural compounds. A potential problem with natural antimicrobials may be that if they are highly purified they would need to be approved as food additives for use as food preservatives. This would involve very expensive and time-consuming toxicological testing.

In addition, they likely would have to be labeled as chemical additives. Of course labeling in this manner would defeat the purpose of using a natural compound. Thus, less purification probably is 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 approval for use. This is possible only if the product from which the extract is taken is known to be nontoxic.

An example of how this might work is found in the recent work on bacteriocins. These compounds may be introduced in foods in three ways: (1) using cultures in a fermented product that produce the compound, (2) using the fermentation medium that the microorganisms was grown in and that contains the antimicrobial, or (3) isolating and purifying the bacteriocin from the growth medium. The first two methods seem to have more immediate promise than the third. The purified compounds likely would be treated by regulatory agencies as a food additive and be required to undergo comprehensive toxicological evaluation. In contrast, the spent fermentation medium usually is whey or milk and essentially would be no different from adding nonfat dry milk or whey to a product.

Another major factor needing to be addressed before applying naturally occurring antimicrobials is their potential impact on the sensory characteristics of a food. Many naturally occurring antimicrobials must be used at high concentrations to achieve antimicrobial activity against microorganisms. Obviously, compounds that negatively affect flavor and odor or contribute inappropriate flavors and odors would be unacceptable. For example, many spice extracts have antimicrobial activity but, at the concentration required for antimicrobial activity, would cause a food to be inedible to most consumers. In addition to adverse effects on flavor, odor, or texture, it would be unacceptable for a food antimicrobial to mask spoilage, as this could protect consumers from ingesting foodborne pathogens.

As indicated, there are a number of potential methods of incorporating naturally occurring antimicrobials in foods. Processors may add antimicrobials to food directly to the formulation or by spraying, immersing, or dusting the product. Antimicrobials may be applied to the packaging material that comes in contact with

the food or incorporated into plastic films used for packaging. As stated previously, the application method selected should reflect antimicrobial solubility and volatility. Very often, the method used is dictated by existing processing and packaging procedures. Bacteriocins or fermentation products are something of a unique case. As discussed, incorporation may include adding a bacteriocin-producing culture to ferment a food product, adding a cultured product, e.g., whey or milk, produced with a bacteriocin-producing strain to another food, or adding a purified bacteriocin to a food.

Perhaps the greatest roadblock to the use of naturally occurring antimicrobials may be their cost. For example, the only antimicrobial enzymes currently produced at a cost to be useful in food preservation are lysozyme and glucose oxidase (Fuglsang et al., 1995). A potential antimicrobial must pay for itself by extending shelflife and/or minimizing the chances of foodborne illness. Depending on the perishability of a food product, even an additional 2 to 3 days of shelflife can offset the cost of an antimicrobial significantly (Branen, 1993).

To summarize, an *ideal* naturally occurring antimicrobial would be effective enough to be added as a whole food or as an edible component, e.g., an herb or a spice. Few, if any, antimicrobials are present in foods at concentrations great enough to be antimicrobials without purification or concentration. Often, even if purification of antimicrobials is possible, their addition to another food may lead to undesirable sensory changes. The ultimate challenge is to find a naturally occurring antimicrobial that can be added to a "microbiologically-sensitive" food product in a nonpurified form from another nonsensitive food. The nonpurified food would have to contain an antimicrobial that is completely nontoxic and highly effective in controlling the growth of microorganisms. For many natural antimicrobials, this well may be impossible. Beuchat and Golden (1989) may have summarized the situation best when they stated that "the challenge is to isolate, purify, stabilize, and incorporate natural antimicrobials into foods without adversely affecting sensory, nutritional, and safety characteristics. . ." and ". . . without increased costs for formulation, processing or marketing."

7 Conclusions

The systems and approaches used in preserving food products have been changing and will continue to change. Consumers prefer minimal processing, absence of synthetic chemicals, high quality, long shelf-life, and safe and convenient products. These preferences should encourage exploitation of naturally occurring antimicrobials. The trend for products with fewer chemical additives and minimal processing already has started. Of the many natural antimicrobial systems or components discussed, however, only a few have been exploited, tested, or applied.

For more extensive use of natural antimicrobial systems, there is a need for research to examine the extraction, isolation, safety, efficacy, interaction with food components and other preservative systems, action mechanisms, application methods, and product quality influences, e.g., nutritional and sensory. Increased knowledge, through research on all aspects of natural antimicrobials, is needed to make effectively designed and safe food preservative systems possible. Because natural compounds with antimicrobial actions are found in combination with other parameters in a multifactor biopreservative system, their interactions with food components or other added compounds need special examination. In addition, care should be taken to ensure that any natural antimicrobial system applied on a commercial scale is safe, addresses a real need, and consists of readily available and economical components that have no adverse effects on food quality. Furthermore, it should be realized that certain natural antimicrobial systems may be appropriate only for certain populations, in which they can aid efforts to address world food supply prob-

lems; even then, however, social/ethical concerns should be taken into account before an application is endorsed.

Additional advances needed for the application of natural antimicrobial systems in food preservation include development of technologies for introduction or delivery of the antimicrobials into foods under various conditions of product manufacture. Development and transfer of such technologies will require investment and involvement of organizations supplying expertise in science, engineering, and economics.

In summary, naturally occurring antimicrobial agents are abundant in the environment. They are present in plant and animal systems or produced by microorganisms. Their antimicrobial activity has been demonstrated in model as well as in actual food systems, and some naturally occurring antimicrobials have found commercial application in food preservation. Their potential for expanded use is positive, especially in light of consumer demands for minimally processed safe foods of adequate shelf-life and convenience and the need for a globally increasing food supply. For the use of naturally occurring antimicrobials to increase, commercial development and economic production must occur on an adequate scale, sensory changes in foods must be avoided, food safety/toxicity must be assessed, multifactor preservative systems including natural antimicrobials must be developed, and technology transfer must occur. With economical preservation systems based on natural antimicrobials, the world will have an important weapon in the struggle against hunger.

Appendix A: Symbols, Acronyms, and Abbreviations

Å	Angstrom	kb	kilobase
ADI	Acceptable Daily Intake	kDa	kiloDalton
BHI	Brain Heart Infusion	L	liter
CDC	Centers for Disease Control and Prevention	LPS	lipopolysaccharide
Da	Dalton	µg	microgram
DNA	deoxyribonucleic acid	µM	micromolar
EDTA	ethylenediaminetetraacetic acid	mg	milligram
Eh	oxidation-reduction potential	min	minute
FAO	Food and Agriculture Organization	M	molar
FDA	U.S. Food and Drug Administration	mL	milliliter
FSIS	U.S. Food Safety and Inspection Service	MW	molecular weight
GMP	good manufacturing practices	pKa	dissociation constant
GRAS	generally recognized as safe	RNA	ribonucleic acid
HACCP	hazard analysis critical control point	UHT	ultra-high temperature
HOCl	hypochlorous acid	USDA	U.S. Department of Agriculture
IU	international unit	WHO	World Health Organization of the United Nations

Appendix B: Glossary

Acidification. Addition of acid to produce a food with a final pH of 4.60 or less.

Antibiotic. A substance produced by certain microorganisms that is antagonistic against other microorganisms.

Antimicrobial. A chemical substance or other agent, natural or synthetic, that inhibits growth of microorganisms.

Antioxidant. A substance which inhibits oxidation of other substances, such as lipids and pigments.

Antiseptic. A substance which inactivates or prevents survival and growth of microorganisms.

Aseptic. Free of spoilage or pathogenic microorganisms.

Disinfectant. A chemical or other agent used to inactivate vegetative cells of undesirable microorganisms.

Drying/Dehydration. Removal of moisture from a food.

Enzyme. A protein compound formed by living cells and acting as an inducer or catalyst of chemical reactions in living organisms or foods.

Fermentation. The anaerobic decomposition of sugars by microorganisms to yield carbon dioxide and water.

Fermented food. A food product made or modified by fermentation.

Food irradiation. Treatment of a food with high energy electromagnetic radiation to inactivate microorganisms.

Food preservative. A chemical agent used to prevent, delay, or inhibit chemical or microbiological food deterioration or development of disease causing microorganisms or toxins.

Food preservation. Use of physical processes, such as heat or radiation, or chemical agents, such as antimicrobials or preservatives, to prevent, inhibit, or delay food deterioration, spoilage, or development of disease causing microorganisms.

Foodborne illness. An illness caused by ingestion of food contaminated with disease causing microorganisms or toxins.

GRAS. Substances classified by the United States Food and Drug Administration as "generally recognized as safe" for use as additives in foods.

Inhibition of microorganisms. Arrest of growth of microorganisms.

Pathogenic. Disease-causing.

Shelf life. The period of time during which a food is safe and acceptable for human consumption.

Spoilage. Loss of desirable or acceptable qualities.

Sterilant. An agent that can inactivate or kill cells and spores of microorganisms.

Water activity. A measure of the free moisture in a food that determines the ability of microorganisms to exhibit metabolic activity.

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Index

A

Abaecin, 74
Acceptable daily intakes (ADI), 10, 21
Acetic acid as food preservative, 18, 19, 67
Acetones, 67
Acidification, definition of, 82
Acidity, 66
Acids, 15, 16
Acipic acid, 23
Adhumulone, 39
Adlupulone, 39
Aflatoxin, 40
 production of, 38, 70
Agriculture, U.S. Department of, Food Safety and Inspection Service, 7
Ajoene, 33
Alkaline phosphatase, 33
Allicin, 4
Allium, 4, 32–35
Allspice, 4, 36
Allyl isothiocyanate, 70–71
Amidases, 5
Anthocyanins, 37, 38
Antibiotics, 15
 definition of, 82
Antimetabolites, 57
Antimicrobial agents, in insects, 58–60
Antimicrobial hurdles, interactions of, 66
Antimicrobial peptides, 53–60
Antimicrobial plant substances, 28–42, 69–71
Antimicrobials, 15–65, 66. *See also* Naturally occurring antimicrobials
 definition of, 82
 factors influencing, 15
 impact of, on microbial growth, 16–17
 lipid, 25–28, 68–69
 miscellaneous microbial metabolites, 60–65, 74–76
 organic acid, 18–25, 67–68
 in plant substances, 28–42, 69–71
 polypeptide, 42–60, 71–74
 role and need for, in food, 12–14
 traditional, 17–18, 67
Antioxidants, 15
 definition of, 82
Antiseptic, definition of, 82
Apidaecin, 74
Ascorbic acid, 24
Aseptic, definition of, 82
Aspergillus, 18
Atmosphere, 66
Attacins, 74
Auberginone, 41, 42

B

Bacteriocins, 5, 53–57, 71, 78, 79
 as natural food antimicrobial, 74
Bacteriolytic enzymes, 5
Barrier concept, 6, 16
Bay leaf, antimicrobial properties of, 70
Benzenecarboxylic acid, 19
Benzoate as food preservative, 20, 67–68
Benzoic acid, 19–20, 77–78
Biopreservation of foods by enzymes, 71
Bovine milk, 5
Branched-chain and hydroxylated fatty acids, 25–26

C

Caffeic acid, 38, 39
Caffeine, 4, 40–41
Calcium ions, 26
Calcium propionate, 68
Caprylic acid, 24
Capsidiol, 41, 42
Carbohydrates, 41
Carrot juice, lethal effect of, 70
Caseins, 5–6
Catalase, 44, 76
Catechin, 32
Cationic peptides, 50, 74
Cationic proteins, 4
Cecropins, 74
Cellosyl, 47
Charcoal, 26
Chemical additives, 13
Chemical additives
 approval and use of, 3
 as food preservatives, 10
Chemical agents exhibiting antimicrobial activity, 1
Chemical composition, 66
Chinese medicinal plants, 70
Chitinases, 5, 48
Chlorogenic acid, 32, 38
Chlorophylls *a* and *b*, 38
Cholesterol, 26
Chromone, 42
Cinnamic acid, 38–39
Cinnamic aldehyde, 34, 35
Cinnamon, antimicrobial properties of, 4, 35, 70
Cis-unsaturated acids, 26
Citric acid, 18, 24
Citrinin, 40
Citrol, 42
Citronellol, 36
Clostridium botulinum, 17, 18

Cloves, antimicrobial properties of, 4, 36, 70
 Cocoa, 40–41
 Coffee, 40–41
 Cohumulone, 39
 Colupulone, 39
 Conalbumin, antibacterial effect of, 5, 51, 72–73
 Consumer perceptions, on naturally occurring antimicrobials, 1, 3–4
 Convenience foods, 14
 Cyanidin, 38

D

D-amino oxidase, 50
 Defensin peptides, 74
 Defensins, 5, 58
 Dehydration, definition of, 82
 Delphinidin, 38
 Dextranocins, 56
 Diacetyl, 63–64
 Dihydroisocoumarin, 42
 Diphenol oxidase enzymes, 32
 Diplococin, 55
 Direct antimicrobial food additives, 15
 Direct food acidification, 16
 Direct microbial inhibition, 17
 Disinfectant, definition of, 82
 Dissociation constant, 78
 DOPA, 32
 Dricin, 55
 Drying, definition of, 82

E

Eggs, 1
 Egg-white lysozyme, 2, 44–47, 71–72
 Emulsifiers, 25
 Endochitinases, 48
 Endopeptidases, 5
 Environmental contaminants, 13
 Enzymatic preservation, 71
 Enzyme, definition of, 82
 Epicatechin, 32
 Erythorbic acid, 18
 Esterification of fatty acids, 26–28
 Esters of *p*-hydroxybenzoic acid, 15–16
 Ethanol, 2, 61–62
 Ethyl acetate, 34
 Ethylenediaminetetraacetic acid (EDTA), 5, 15, 45–46
 Eugenol, 36

F

Falcarindiol, 6-methoxymellein, 41
 Fatty acid esters, 26–28
 Fatty acids, 25–26
 Fermentation
 definition of, 82
 food, 1, 17, 74, 82
 meat, 37
 Ferrous/ferric ions, 51
 Ferulic acid, 38
 Flat-sour spoilage, 24

Flavanols, 38
 Food additives, 1, 7, 10, 78–79
 Food Additives Amendment of the Food, Drug and Cosmetic Act, 10
 Food and Agriculture Organization (FAO), 10, 21
 Food and Drug Administration (FDA), 7, 10, 14
 Food associated fungi, 18
 Foodborne illness, 13
 definition of, 82
 Food fermentation, 1, 17, 74, 82
 Food irradiation, definition of, 82
 Food preservation, 3, 9
 concept of, 66
 definition of, 82
 importance on, 3
 important systems of, 9–10
 need for, 1
 Food preservatives, 78
 definition of, 10, 82
 Food(s)
 activity and uses of naturally occurring antimicrobials in, 67–76
 as necessity, 9
 role and need for antimicrobials in, 12–14
 Food safety, importance of, 1
 Fructose, 17
 Fruits, 1
 Fumaric acid, 18, 24, 69
 Fungi, control of, 69

G

Garlic, antimicrobial properties of, 4, 29, 32–35, 70
 Generally recognized as safe (GRAS), 10, 20, 21, 23, 24, 25, 62
 definition of, 82
 Geraniol, 36, 42
 Glucose, 17
 Glucose oxidase, 7, 44, 50, 72
 Glucosinolates, 31
 Glyceollin, 41
 Glycerol, 41–42
 Glycosidases, 5
 Good manufacturing practices (GMP), 10, 23

H

Hazard analysis critical control point (HACCP), 1, 66
 Hen egg white lysozyme, 44–47, 71–72
 Herbs, 1
 phenolic compounds in, 35–37
 Heterofermentative organisms, 20–21
 Hops, 29, 39–40
 Humulone, 39
 Hurdle concept, 6, 16
 Hydrogen peroxide, 2, 4, 48, 64–65, 76
 Hydrophobic groups, 26
 Hydrophobicity, 78
 Hydroxycinnamic acid, 38–39
 Hydroxysuccinic acid, 24
 Hypochlorous acid, 50

I

Indirect antimicrobial agents, 15
 Inhibitins, 29
 Inhibition
 magnitude of, 26
 of microorganisms, 82
 Insects, antimicrobial agents in, 58–60
 In vitro testing, 77
 Ipomeamarone, 41
 Isothiocyanates, 31, 70–71

K

Killer toxins, 5, 58
 Kola, 40–41

L

Lactic acid, 18, 20–21, 64
 fermentation, 15
 as food preservative, 68
 Lactic acid bacteria, 1, 18
 nonprotein inhibitors from, 63–65
 Lactic dehydrogenase, 33
 Lactocins, 55
Lactobacillus spp., 39–40
 Lactococcins, 55
 Lactoferrin, 51–52
 Lactoferrin, antibacterial effect of, 5, 14, 48, 50, 51–52, 71, 72–73
 Lactoperoxidase, 5, 14, 48–49, 51, 72
 Lactoperoxidase/thiocyanate/hydrogen peroxide system, 72
 Lactostrepcins, 55
 Lauric acid, 25, 27
 Lecithin, 26
 Leek, 4
 Limonene, 42
 Linden flower, 41
 Linoleic acid, 26
 Linolenic acid, 26
 Lipases, 4, 5
 Lipid antimicrobials, 25–28, 68–69
 Lipids, 17
 Lipopeptides, 5, 28
 Lipophilic compounds, 77
 Lipopolysaccharides (LPS), 5, 17, 25
Listeria monocytogenes, 18
 Long-chain fatty acids, 25
 Lubimin, 42
 Lysis, 4
 Lysostaphin, 48
 Lysozyme, 4, 7, 14, 44, 51, 71–72, 77
 egg-white, 2, 44–47, 71–72
 Lytic enzymes, 4, 44–48

M

Mace, antimicrobial properties of, 70
 Magnesium ions, 26
 Maillard reaction, 46
 Malic acid, 18, 24
 Malnutrition, 13
 Malvidin, 38

Meat fermentation, 37
 Medium-chain saturated fatty acids, 25
 Mesenterocins, 56
 Metal-binding polypeptides, 52–53
 Metallothioneins, 52–53
 Microbial control, 66
 Microbial foodborne illness, 3
 Microbial growth, impact of antimicrobials on, 16–17
 Microbial metabolites, with antimicrobial activity, 60–65, 74–76
 Microbiologically-sensitive food, 7
 Microorganisms
 control of, 66
 factors influencing physiology of, 66
 as food hazards, 13
 Milk, 1
 bovine, 5
 Molds, 18, 22
 Monoacylglycerols, 26–28, 69
 Monocaprin, 69
 Monocaprylglycerol, 69
 Monomyristylglycerol, 27
 Monopalmitylglycerol, 27
 Monounsaturated fatty acids, 18–19
 Mosquitoes, activities of garlic against, 34
Mucor, 18
 Multifactor food preservation system, search for, 66
 Mustard, antimicrobial properties of, 70
 Mutanolysin, 48
 Myeloperoxidase, 50

N

Natamycin, 2, 62–63, 75–76
 National Food Safety Initiative, 13
 Natural antimicrobial agents, 14
 Natural foods, 1, 4
 acidification, 16
 Naturally occurring antimicrobials, 1, 3
 activity and uses of, in foods, 67–76
 consumer perceptions of, 1, 3–4
 future of, 2
 potential for applications of, 77–79
 Natural sugars, 17
 Natural toxins, 13
 Nisin, antimicrobial activity of, 2, 4, 55–56, 73–74, 77
 Nonprotein inhibitors from lactic acid bacteria, 63–65
 Nutmeg, antimicrobial properties of, 70

O

Ochratoxin A, 40
o-coumaric acid, 39
 Octanoic acid, 24
 Oleic acid, 26
 Oleoresins, 36
 Olive oil, 70
 Onion, antimicrobial properties of, 4, 32–35, 70
 Oregano, 37
 Organic acids, 1
 as antimicrobials, 18–25, 67–68
 Ovotransferrin, 5, 14, 51
 Oxidases, 5, 44, 48–50
 Oxygen-independent defense mechanisms, 4

P

Palmitoleic acid, 26, 27
 Papaverine, 40
 Pathogenic, definition of, 82
 Patulin, 40
p-coumaric acid, 38
 Pediocin A, 56–57
 Pediocin AcH, 56–57
 Pelargonidin, 38
Penicillium, 18
 Peracetic acid, as food preservative, 67
 Peroxidases, 5, 44, 48–50
 Pesticides, 13
 Petunidin, 38
 pH, 18
 Phaseollin, 41
 Phenolic antioxidants, 15
 Phenolic compounds, 4, 37, 38–39, 67, 70
 in herbs, 35–37
 in spices, 35–37
 Phenylformic acid, 19
 Phenyl rings, 26
 Phosphates, 15
 Phytoalexins, 4, 29, 41, 42
 Phytuberin, 42
 Pisatin, 41
 pKa, 78
 Plant phenolic compounds, 37, 38–39
 Plant pigments, 37–38
 Plants, antimicrobial agents present in, 4
 Plant substances
 antimicrobial, 28–42, 69–71
 Polyketide mycotoxin synthesis, 40
 Polymyxins, 28
 Polyoxyethylene esters, 27
 Polypeptides, 4, 60
 as antimicrobials, 42–60, 71–74
 Posthumulone, 39
 Postinhibitins, 29
 Postlupulone, 39
 Potassium salt, 68
 Potassium sorbate, 23
 Prehumulone, 39
 Prelululone, 39
 President's National Food Safety Initiative, 1
 Proanthocyanidins, 38
 Prohibitins, 29
 Propionates, as food preservative, 21, 68
 Propionic acid, 21
 Proteases, 4
 Proteins, 17
 Psychrotrophic bacteria, 13

Q

Quinic acid, 38
 Quinones, 32

R

Resins, 39–40
 Resistant strains, development of, 15

Resveratrol, 41
Rhizopus, 18
 Rimocidin, 76
 Rishitin, 41, 42
 Rosemary, antimicrobial properties of, 37, 70
 Rosemary spice extract, 70

S

Safynol, 41
 Sage, antimicrobial properties of, 70
 Salts, 15, 16, 17–18
 Saponin, 26
 Saturated fatty acids, 26
 Scopoletin, 42
 Serotransferrin, 51
 Shelf life
 definition of, 82
 extending, 67, 69, 72
 Short-chain fatty acids, 25
 Short-chain organic acids, 18
 Smoking of foods, 18
 Sodium benzoate, 19
 Sodium lactate, as food preservative, 68
 Sodium propionate, 68
 Sorbates, 22, 68
 Sorbic acid, 15, 22–23, 77–78
 Sorbic acid benzoic acids, 16
 Spices, 1, 28–29
 phenolic compounds in, 35–37
 Spoilage, definition of, 82
Staphylococcus aureus, 18
 Sterigmatocystin, 40
 Sterilant, definition of, 82
 Sublethal injury, 77
 Succinic acid, 18, 24–25
 Succrose polyesters, 27
 Sucrose, 17, 27
 Sulfhydryl enzymes, 32–33
 Superoxide ions, 4
 Syrups, 17

T

Tannic acid, 39
 Tannins, 39
 Tartaric acid, 25
 Tea, 40–41
 Temperature, 66
 Teriochoic acid, 44
 Terpinene, 42
 Terpeneol, 42
 Theobromine, 4, 41
 Theophylline, 4, 40–41
 Thiocyanate, 48, 72
 Thyme, 37
 Thymol, 36
 Traditional antimicrobials, 17–18, 67
 Transferrins, 50–53
Trans-unsaturated fatty acids, 26
Trichoderma, 12
 Turmeric, 37
 Tyrosinase, 33



ISBN 1-887383-12-3