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Application of Natural Antimicrobials for Food Preservation

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In this review, antimicrobials from a range of plant, animal, and microbial sources are reviewed along with their potential applications in food systems. Chemical and biochemical antimicrobial compounds derived from these natural sources and their activity against a range of pathogenic and spoilage microorganisms pertinent to food, together with their effects on food organoleptic properties, are outlined. Factors influencing the antimicrobial activity of such agents are discussed including extraction methods, molecular weight, and agent origin. These issues are considered in conjunction with the latest developments in the quantification of the minimum inhibitory (and noninhibitory) concentration of antimicrobials and/or their components. Natural antimicrobials can be used alone or in combination with other novel preservation technologies to facilitate the replacement of traditional approaches. Research priorities and future trends focusing on the impact of product formulation, intrinsic product parameters, and extrinsic storage parameters on the design of efficient food preservation systems are also presented.

KEYWORDS: Antimicrobial activity; chemical compounds; plant/animal/microbial antimicrobials mechanism; minimum inhibitory concentration

INTRODUCTION

A number of nontraditional preservation techniques are being developed to satisfy consumer demand with regard to nutritional and sensory aspects of foods. Generally, foods are thermally processed by subjecting them to temperatures varying from 60 to 100 °C for the duration of a few seconds to a minute in order to destroy vegetative microorganisms. During this period of treatment, a large amount of energy is transferred to the food. However, this energy can trigger unwanted reactions, leading to undesirable organoleptic and nutritional effects (*1*). Ensuring food safety and at the same time meeting such demands for retention of nutrition and quality attributes has resulted in increased interest in alternative preservation techniques for inactivating microorganisms and enzymes in foods. Quality attributes of importance include flavor, odor, color, texture, and nutritional value. This increasing demand has opened new dimensions for the use of natural preservatives derived from plants, animals, or microflora. In biopreservation, storage life is extended, and/or safety of food products is enhanced by using natural or controlled microflora, mainly lactic acid bacteria (LAB) and/or their antibacterial products such as lactic acid, bacteriocins, and others (*2*). Typical examples of investigated compounds are lactoperoxidase (milk), lysozyme (egg white, figs), saponins and flavonoids (herbs and spices), bacteriocins (LAB), and chitosan (shrimp shells) (*3*). Antimicrobial compounds present in foods can extend the shelf life of unprocessed or processed foods by reducing the microbial growth rate or viability (*4*). Originally, spices and herbs were added to change or to improve taste. Some of these substances are also known to contribute to the self-defense of plants against infectious organisms (*5*, *6*).

Extensive research has investigated the potential application of natural antimicrobial agents in food preservation. In this review, antimicrobials and their chemical and biochemical components from a range of natural sources and their applications in food systems are reviewed. Natural antimicrobials in food preservation can be used alone or in combination with other nonthermal technologies. Naturally derived antimicrobial systems from plant, animal, and microbial origin are detailed, and the latest developments in the quantification of the minimum (and noninhibitory) concentration of antimicrobials and/or their components are presented.

PLANT ORIGIN ANTIMICROBIAL AGENTS

Edible, medicinal, and herbal plants and their derived essential oils (EO) (and their hydrosols, i.e., byproducts of an essential oil purification procedure) and isolated compounds contain a large number of secondary metabolites that are known to retard or inhibit the growth of bacteria, yeast, and molds (*7*, *8*). Many of these compounds are under investigation and are not yet exploited commercially. The antimicrobial compounds in plant materials are commonly found in the essential oil fraction of leaves (rosemary, sage, basil, oregano, thyme, and marjoram), flowers or buds (clove), bulbs (garlic and onion), seeds (caraway,

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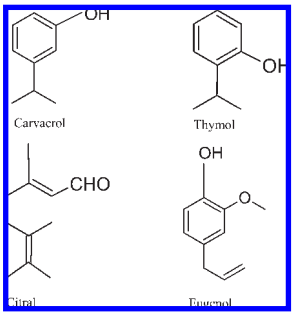
Scheme 1. Plant Origin Antimicrobial Agents



fennel, nutgem, and parsley), rhizomes (asafetida), fruits (pepper and cardamom), or other parts of plants (*9*, *10*). Plant EOs and their constituents have been widely used as flavoring agents in foods since the earliest recorded history, and it is well established that many have a wide spectra of antimicrobial action (*11*−*15*). These compounds may be lethal to microbial cells or they might inhibit the production of secondary metabolites (e.g., mycotoxins) (*16*). Plant essential oils are generally more inhibitory against Gram-positive than Gram-negative bacteria (*10*, *17*, *18*). While this is true for many EOs, there are some agents that are effective against both groups, such as oregano, clove, cinnamon, and citral (*19*−*21*). The major EO components with antimicrobial effects found in plants, herbs, and spices are phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids (*8*, *22*−*27*). Chemical analysis of a range of EOs revealed that the principal constituents of many include carvacrol, thymol, citral, eugenol (see **Scheme 1** for their chemical structure), and their precursors (*8*, *28*−*30*). It has been reported that some nonphenolic constituents of EOs are more effective or quite effective against Gram-negative bacteria, e.g., allyl isothiocyanate (AIT) (*31*) and garlic oil (*32*), respectively. In addition, AIT is also effective against many fungi (*33*). Generally, the antimicrobial efficacy of EOs is dependent on the chemical structure of their components as well as the concentration. Many of the antimicrobial compounds present in plants can be part of their pre- or postinfectional defense mechanisms for combating infectious or parasitic agents (*34*). Consequently, plants that manifest relatively high levels of antimicrobial action may be sources of compounds that inhibit the growth of foodborne pathogens (*35*). Compounds are also generated in response to stress from inactive precursors (*36*), which may be activated by enzymes, hydrolases or oxidases, usually present in plant tissues (*37*). In mustard and horse radish, precursor glucosinolates are converted by enzyme myrosinase to yield a variety of isothiocynates including the allyl form, which is a strong antimicrobial agent (*38*).

The application of plant EOs for controlling the growth of foodborne pathogens and food spoilage bacteria requires evaluation of the range of activity against the organisms of concern to a particular product, as well as effects on a food's organoleptic properties. Plant EOs are usually mixtures of several components. Oils with high levels of eugenol (allspice, clove bud and leaf, bay, and cinnamon leaf), cinnamamic aldehyde (cinnamon bark and cassia oil), and citral (lemon myrtle, *Litsea cubeba*, and lime) are usually strong antimicrobials (*39*, *40*). The EOs from *Thymus* spp. possess significant quantities of phenolic monoterpenes and have reported antiviral (*41*), antibacterial (*42*, *43*), and antifungal (*44*, *45*) properties. The volatile terpenes carvacrol, *p*-cymene, γ-terpinene, and thymol contribute to the antimicrobial activity of oregano, thyme, and savory (*18*). The antimicrobial activity of sage and rosemary can be attributed to borneol and other phenolic compounds in the terpene fraction. Davidson and Naidu (*40*) reported that the terpene thejone was responsible for the antimicrobial activity of sage, whereas in rosemary, a group of terpenes (borneol, camphor, 1,8 cineole, *a*-pinene, camphone, verbenonone, and bornyl acetate) was responsible. Plant EOs such as cumin, caraway, and coriander have inhibitory effects on organisms such as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, and *Staphylococcus aureus* (*46*, *47*), marjoram and basil have high activity against *B. cereus*, *Enterobacter aerogenes*, *Escherichia coli*, and *Salmonella*, and lemon balm and sage EOs appear to have adequate activity against *L. monocytogenes* and *S. aureus* (*10*). Gutierrez et al. (*10*) showed that oregano and thyme EOs had comparatively high activity against enterobacteria (minimum inhibitory concentration (MIC) of oregano and thyme at a range of 190 ppm and 440 ppm, respectively, for *E. cloacae*), lactic acid bacteria (MIC of oregano and thyme at a range of 55 ppm and 440 ppm, respectively, for *Lactobacillus brevis*), *B. cereus* (MIC of oregano and thyme at a range of 425 ppm and 745 ppm, respectively), and *Pseudomonas* spp (MIC of oregano and thyme at a range of 1500 ppm for *P. putida*), although in general *Pseudomonas* species are consistently highly resistant to plant antimicrobials (*10*, *48*). One of the attributed factors can be the production of exopolysaccharide layers forming biofilms of the microorganism that can delay penetration of the antimicrobial agent (*49*).

Lee et al. (*50*) investigated the antibacterial activity of vegetables and juices and concluded that green tea and garlic extracts have broad applications as antibacterial agents against a wide range of pathogens. Arrowroot tea extract has reported antimicrobial activity against *E. coli* O157:H7 (*19*). Ibrahim et al. (*35*) reported the potential of caffeine at a concentration of 0.5% or higher as an effective antimicrobial agent for the inactivation of *E. coli* O157:H7 in a liquid system (i.e., brain heart infusion (BHI)).

Mechanisms of Antimicrobial Action. The possible modes of action for phenolic compounds (EO fractions) as antimicrobial agents have been previously reviewed (*16*, *24*, *27*, *36*, *51*−*53*). However, the exact mechanism of action is not clear. The effect of phenolic compounds can be concentration dependent (*54*). At low concentration, phenols affect enzyme activity, particularly those associated with energy production, while at high concentrations, they cause protein denaturation. The antimicrobial effect of phenolic compounds may be due to their ability to alter microbial cell permeability, thereby permitting the loss of macromolecules from the interior (for example ribose and Na glutamate) (*55*). They could also interfere with membrane function (electron transport, nutrient uptake, protein, nuclein acid synthesis, and enzyme activity) (*55*) and interact with membrane proteins, causing deformation in structure and functionality (*56*−*58*). The high antibacterial activity of phenolic components can be further explained in terms of alkyl substitution into the phenol nucleus (*25*). The formation of phenoxyl radicals that interact with alkyl substituents does not occur with more stable molecules such as the ethers myristicin or anethole, which was related to the relative lack of antimicrobial activity of fennel, nutmeg, or parsley EOs (*10*).

Delaquis and Mazza (*38*) reported that the antimicrobial activity of isothiocynates derived from onion and garlic is related to the inactivation of extracellular enzymes through oxidative cleavage of disulfide bonds and that the formation of the reactive thiocyanate radical was proposed to mediate the antimicrobial effect. Carvacrol, (+)-carvone, thymol, and *trans*-cinnamaldehyde are reported to decrease the intracellular ATP (adenosine triphosphate) content of *E. coli* O157:H7 cells while simultaneously increasing extracellular ATP, indicating the disruptive action of these compounds on the plasma membrane (*59*). Inactivation of yeasts can be attributed to the disturbance of several enzymatic systems, such as energy production and structural component synthesis (*60*).

Factors Affecting Antimicrobial Activity. Antimicrobial activity of EOs is influenced by a number of factors including botanical source, time of harvesting, stage of development, and method of extraction (*61*). For example, Chorianopoulos et al. (*62*) reported that *Satureja* EOs obtained during the flowering period were the most potent with bactericidal properties. The composition, structure as well as functional groups of the oils play an important role in determining their antimicrobial activity. Usually compounds with phenolic groups are the most effective (*5*, *25*). Most studies related to the antimicrobial efficacy of EOs have been conducted in vitro using microbiological media (*63*−*71*). Consequently, there is less understanding related to their efficacy when applied to complex food systems. Key areas requiring further knowledge for optimized application of natural antimicrobials in food include targeting the microorganism of concern, the intelligent use of combinations to provide a synergy of activity, matching the activity of the compounds to the composition, and processing and storage conditions of the food (*9*, *72*).

Plant EOs of thyme, clove, and pimento were tested against *Listeria monocytogenes* and were found to be highly effective in peptone water. However, when the EOs were applied in a food system, Singh et al. (*73*) concluded that efficacy of EOs was reduced due to interaction with food components. In general, higher concentrations of EOs are required in foods than in laboratory media. Combinations of EOs could minimize the application concentrations required, thereby reducing any adverse organoleptical impact; however, their application for microbial control may also be affected by food composition (*74*). The antimicrobial efficacy of EOs was found to be a function of ingredient manipulation, for example, the antimicrobial activity of thyme is increased in high protein concentrations, concentrations of sugars above 5% on the microbial growth medium did not reduce EO efficacy, and high potato starch concentrations decreased the EO antimicrobial activity of oregano and thyme on *L. monocytogenes* in food model systems (*74*, *75*). Finally, low pH values (of the range of 5) seemed to have the highest impact on the increase of the antimicrobial effect of EOs on *L. monocytogenes* (*74*). Low pH values appear to increase the hydrophobicity of EOs, consequently enabling easier dissolution in the lipids of the cell membrane of target bacteria (*54*).

Accordingly, the challenge for practical application of EOs is to develop optimized low dose combinations to maintain product safety and shelf life, thereby minimizing the undesirable flavor and sensory changes associated with the addition of high concentrations of EOs.

ANIMAL ORIGIN ANTIMICROBIAL AGENTS

There are numerous antimicrobial systems of animal origin, where they have often evolved as host defense mechanisms. Lysozyme is a bacteriolytic enzyme, commercially sourced from hen's egg white which is reported to inhibit the outgrowth of *Clostridium tyrobutyricum* spores in semihard cheeses (*76*). Lysozyme has found commercial applications; inovapure is said to be effective against a wide range of food spoilage organisms and can be successfully used to extend the shelf life of various food products, including raw and processed meats, cheese, and other dairy products. The lactoperoxidase system, which is naturally active in milk, has strong antimicrobial effects against both bacteria and fungi. A wide range of both Gram-negative (*77*) and Gram- positive bacteria (*78*) are inhibited by the lactoperoxidase system. However, studies have shown that Gram-negative bacteria were generally found to be more sensitive to lactoperoxidase mediated food preservation than Gram-positive species (*79*, *80*). Many of the antimicrobial agents inherent to animals are in the form of antimicrobial peptides (polypeptides).

Antimicrobial peptides were first isolated from natural sources in the 1950s when nisin was isolated from lactic acid bacteria for potential application as a food preservative (*81*). Subsequently, antimicrobial peptides were isolated from other natural sources, such as plants, insects, amphibians, crustaceans, and marine organisms (*82*−*84*). Antimicrobial peptides (AMPs) are widely distributed in nature and are used by many if not all life forms as essential components of nonspecific host defense systems. The list of discovered AMPs has been constantly increasing, with much discovery in the last two decades. The list of AMPs produced by animal cells includes magainin (*85*), MSI-78 (*86*), PR-39 (*87*), spheniscin (*88*), pleurocidin (*89*), dermaseptin S4 (*90*), K4S4-(1-14) (*91*), cecropin P1 (*92*), melittin (*93*), LL-37 (*94*), clavanin A (*92*), and curvacin A (*95*). Antimicrobial peptides present a promising solution to the problem of antibiotic resistance because, unlike traditional antimicrobial agents, specific molecular sites are not targeted, and their characteristic rapid destruction of membranes does not allow sufficient time for even fast-growing bacteria to mutate. Some of the potential antimicrobials of animal origin which could be used as food additives are discussed below.

Pleurocidin. Pleurocidin, a 25 amino acid peptide isolated from the skin mucus membrane of the winter flounder (*Pleuronectes americanus*) is active against Gram-positive and Gram-negative bacteria. It is heat-stable, salt-tolerant, and insensitive to physiological concentrations of magnesium and calcium (*96*). Pleurocidin has potential for use in food applications and was found to be effective against foodborne organisms including *Vibrio parahemolyticus*, *L. monocytogenes*, *E. coli* O157:H7, *Saccharomyces cerevisiae*, and *Penicillium expansum* (*97*). The antimicrobial activity of pleurocidin against foodborne microorganisms was reported at levels well below the legal limit for nisin (10,000 IU/g) without significant effect on human red blood cells (*97*), thereby indicating its potential as a food preservative and a natural alternative to conventional chemicals. However, pleurocidin was inhibited by magnesium and calcium (*96*), which may limit the use of this AMP in environments rich in these cations.

Defensins. Defensins are another group of antimicrobial peptides widely found in nature including mammalian epithelial cells of chickens, turkeys, etc. They are abundant in cells and tissues active in host defense against microorganisms (*98*, *99*). They are reported to have a broad spectrum of antimicrobial activity (*100*), including Gram-positive, Gram-negative bacteria, fungi, and enveloped viruses (*101*, *102*, *107*).

Lactoferrin. Bovine and activated lactoferrin (ALF), an iron-binding glycoprotein present in milk, has antimicrobial activity against a wide range of Gram-positive and negative bacteria (*102*) fungi, and parasites (*103*). Lactoferrin has been applied in meat products (*104*−*106*) as it has recently received approval for application on beef in the USA (USDA-FSIS 2008. FSIS Directive 7120.1 Amendment 15).

Other AMPs. Protamine, like salmine and clupeine, has been reported to be isolated from fish and is found to be effective against Gram-negative and Gram-positive bacteria, yeasts, and molds (*108*−*111*). Magainin peptides isolated from frogs (*112*) have been found effective against a range of food-related pathogens (*113*), implying a possible application as food preservatives (*91*, *114*, *115*).

Chitosan. Chitosan, a natural biopolymer obtained from the exoskeletons of crustaceans and arthropods, is known for its unique polycationic nature and has been used as active material for its antifungal activity (*72*, *116*) and antibacterial activity (*117*−*120*). Liu et al. (*121*) studied the efficacy of chitosan against *E. coli* and concluded that low molecular weight chitosan is effective for controlling growth. The strong antibacterial activity of chitosan was also observed against *S. aureus*, while its molecular weight appeared to be a significant parameter defining its activity (*122*).

Lipids. Like lipids of plant origin, lipids of animal origin have antimicrobial activity against a wide range of microorganisms. Free fatty acids at mucosal surfaces have been shown to inactivate *S. aureus* (*123*). Milk lipids have recorded activity for inactivation of Gram-positive bacteria including *S. aureus*, *Cl. botulinum*, *B. subtilis*, *B. cereus*, *L. monocytogenes*, Gram- negative bacteria such as *P. aeruginosa*, *E. coli*, and *Salmonella enteriditis* (*124*−*126*), and also against various fungi such as *Aspergillus niger*, *Saccharomyces cerevisiae*, and *C. albicans* (*36*, *124*). Lipids may serve to inhibit the proliferation as well as the prevention of the establishment of pathogenic or spoilage microorganisms in food matrixes.

Shin et al. (*127*) studied eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are formed in animal (including fish and shellfish) tissues but not plant tissues (18:3 ω-3). DHA is a component of membrane structural lipids that are enriched in certain phospholipid components of the retina and nonmyelin membranes of the nervous system in animals. Bioconverted EPA and DHA exhibited antibacterial activities against four Gram-positive bacteria, *B. subtilis*, *L. monocytogenes*, *Staphylococcus aureu*s ATCC 6538, *S. aureus* KCTC 1916, and seven Gram-negative bacteria, *E. aerogenes*, *E. coli*, *E. coli* O157:H7, *E. coli* O157:H7 (human), *P. aeruginosa*, *Salmonella enteritidis*, and *S. typhimurium* (*127*). The growth inhibition by both EPA and DHA was similar against Gram-positive bacteria, while the bioconverted extract of DHA was more effective than EPA against Gram-negative bacteria.

Mechanism of Antimicrobial Action. The mechanism of action of AMPs seems to involve multiple targets. The plasma membrane is the most cited target; however, recent studies suggest intracellular targets at least for some peptides (*128*, *129*). Although most AMPs act by nonspecific mechanisms, they often display some selectivity between different microorganisms, for example, Gram-negative compared with Gram-positive bacteria (*130*, *131*) and susceptibility of fungal cells compared with other eukaryotic cells (*132*). Antimicrobial peptides can assume amphipathic structures, which are able to interact directly with the microbial cell membrane, rapidly disrupting the membrane in several locations, resulting in leaching out of vital cell components (*96*, *133*). Previous studies conducted on the mechanism of action of pleurocidin revealed that it exhibits strong membrane translocation and pore-formation ability, reacting with both neutral and acidic anionic phospholipid membranes (*134*). Lipids inactivate microorganisms mainly by disruption of bacterial cell wall or membrane, inhibition of intracellular replication, or inhibition of an intracellular target (*135*). Monoacylglycerols lower the heat resistance of certain bacteria and fungi; therefore, they may find application in reducing the required heat treatment for certain foods (*36*). Lysozyme hydrolyses the β-1,4-glycosidic linkage in sugar polymers such as *N*-acetylmuramic acid and *N*-acetylglucosamine linkages found in bacterial peptidoglycan (*136*).

MICROBIAL ORIGIN ANTIMICROBIAL AGENTS

Bacteria produce many compounds that are active against other bacteria, which can be harnessed to inhibit the growth of potential spoilage or pathogenic microorganisms. These include fermentation end products such as organic acids, hydrogen peroxide, and diacetyl, in addition to bacteriocins and other antagonistic compounds such as reuterin (*137*). Both Gram-negative and Gram-positive bacteria produce bacteriocins. Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides (*138*). Bacteriocin production can be exploited by food processors to provide an additional barrier to undesirable bacterial growth in foods (**Table 1**).

Bacteriocins are cationic peptides that display hydrophobic or amphiphilic properties, and in most cases, the target for their activity is the bacterial membrane. Depending on the producer organism and classification criteria, bacteriocins can be categorized into several groups (*139*−*142*) with as many as five classes of bacteriocins proposed (*143*−*145*). The majority fall into classes I and II, which are the most intensively researched to date. The class I group, termed lantibiotics, are small peptides that are characterized by their content of several unusual amino acids (*146*). The class II bacteriocins are small, nonmodified, heat stable peptides (*147*). Another classification is with respect to the producing microorganism and is specifically named after the genus, species, or the group of microorganisms, e.g., lantibiotics for bacteriocins of lactic acid bacteria, colicins of *E. coli*, klebisins of *Klebsiella pneumoniae* (*148*). A large number of bacteriocins have been isolated and characterized from lactic acid bacteria, and some have acquired a status as potential food preservatives because of their antagonistic effect on important pathogens. Many bacteriocins are active against food borne pathogens and spoilage bacteria (*149*−*152*). The important ones include nisin, diplococcin, acidophilin, bulgarican, helveticin, lactacin, and plantaricin (*153*). Nisin is produced by various *Lactococcus lactis* strains, is the most thoroughly studied bacteriocin to date, and is applied as an additive in food worldwide (*154*). While the antimicrobial polypeptide nisin and related compounds such as pediocin are the only bacteriocins widely used for food preservation (*155*, *156*), many other bacteriocins have been reported and have shown potential for food preservation and safety applications.

Reuterin. Reuterin (β-hydroxypropionaldehyde) is a water-soluble nonproteinaceous metabolite of glycerol (*157*). It is a broad spectrum antimicrobial compound produced by some strains of *Lactobacillus reuteri*, with recorded activity against Gram-negative and Gram-positive bacteria, yeasts, and filamentous fungi (*158*). Reuterin was isolated, purified, and identified by Talarico and Dobrogosz (*159*) and is active over a wide range of pH values and resistant to the action of proteolytic and lipolytic enzymes (*160*). Reuterin is reported to exhibit bacteriostatic activity against *Listeria monocytogenes* but was only slightly bactericidal against *Staphylococcus aureus* at 37 °C. However, higher bactericidal activity was reported against *E. coli* O157:H7, *S. choleraesuis* subsp. *Choleraesuis*, *Y. enterocolitica*, *A. hydrophila* subsp. *Hydrophila*, and *C. jejuni* (*161*).

Pediocin. Pediocin is produced by strains of *Pediococcus acidilactici* and *P. pentosaceus* and is designated generally recognized as safe (GRAS). The organism is commonly isolated from and used in fermented sausage production. The bacteriocins produced by *P. acidilactici* are AcH, PA-1, JD, and 5, and those produced from *P. pentosaceus* are A, N5p, ST18, and PD1 (*162*). Most pediocins are thermostable proteins and function over a wide range of pH values. Pediocin AcH has proven efficacy against both spoilage and pathogenic organisms, including *L. monocytogenes*, *Enterococcus faecalis*, *S. aureus*, and *Cl. Perfringens* (*163*). Natamycin is an antifungal produced by *Streptomyces natalensis* that is effective against nearly all molds and yeasts but has little or no effect on bacteria.

Nisin. Nisin is the most widely used bacteriocin. To date, nisin is the only natural antimicrobial peptide (see **Scheme 2** for its structure) approved by the FDA for use as a food preservative; however, it has a limited spectrum of activity, does not inhibit Gram-negative bacteria or fungi, and is only effective at low pH (*164*, *165*). Nisin is produced by fermentation of a modified milk medium by certain strains of lactic acid bacterium, *Lactococcus lactis*. Nisin functions by interacting with the phospholipids

Table 1. Effect of Natural Antimicrobial Agents on Food Preservation and Quality[a]

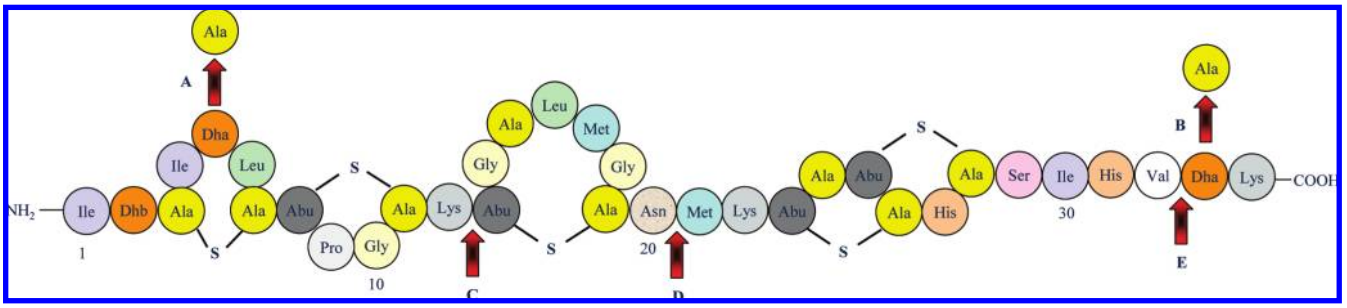
food product	antimicrobial agent (concentrations)	microbial dynamics	quality attributes	reference
fruit yoghurt	vanillin (2000 ppm)	yeast, bacterial (delays growth)	shelf life (↑)	(*232*)
tomato juice	clove oil (0.1%)	total plate count (3.9LR)	shelf life (↑), vitamin C (∼)	(*208*)
	mint extract (1.0%)	total plate count (8.34LR)		
	nisin (0.004%)	total plate count (↓)		
ready-to-eat fruit salad	citral (25−125 ppm)	yeasts and lactic acid bacteria (LAB) (delays growth)	shelf life (↑)	(*233*)
	citron (300−900 ppm)			
	citron (600 ppm)	*Salmonella enteritidis* E4 (2 LR),	sensory characteristics (∼)	
		Escherichia coli 555 (<4.5 LR)		
		Listeria monocytogenes Scott A (4 LR)		
raspberries	methyl jasmonate (MJ),		AC (↑)	(*234*)
	allyl isothiocyanate (AITC)		AC (↓)	
	EO of *Melaleuca alternifolia* (tea tree oil)		AC (↑)	
fresh cut water melon	nisin (25 μg/mL)	*L. monocytogenes* (0.8 LR)	quality (↑)	(*235*)
lettuce	thyme oil (1 mL/l)	*E. coli* (6.32LR)		(*236*)
baby carrot		*E. coli* (5.57LR)		
minimally processed carrots	oregano oil (250 ppm)	background spoilage microflora	sensory characteristics (∼)	(*205*)
		total viable count (TVC) (>1 LR)		
		lactic acid bacteria (LAB) (>1 LR)		
		Pseudomonas (<1 LR)		
minimally processed vegetables	thyme oil (1%)	*Aeromonas spp* (2 LR)	sensory properties (↓), shelf life (↑)	(*237*)
		psyschrotrophic aerobic plate count (4.19 LR)		
		plate count agar (5.44 LR)		
wine	nisin	LAB (minimum inhibitory concentration, MIC = 0.39 mg/mL)		(*238*)
		Oenococcus oeni (MIC 0.01 mg/mL)		
		acetic acid bacteria (MIC 1.5 mg/mL)		
milk	reuterin (8 AU/ml)	*L. monocytogenes* (4.59 LR)		(*161*)
	nisin (100 IU/ml)	*S. aureus* counts (5.45 LR)		
skimmed milk powder	nisin (100 IU/ml)	*L. innocua* (3.8 LR)		(*239*)
chicken meat	nisin	*E. coli* (<1 LR)	proximate composition (∼), shelf life (↑)	(*209*)
		Brochothrix thermosphacta (∼)		
	EOs of mustard oil	*Lactobacillus alimentarius* (∼)		
		Brochothrix thermosphacta (∼)		
		Lactobacillus alimentarius (delays growth)		
fish	EOs (0.5% carvacrol + 0.5% thymol)	TVC (2.5LR)	shelf life (↑), lipid oxidation (↓) sensory characteristics (∼)	(*240*)
red meat	tea catechins (300 mg/kg)		shelf life (↑), lipid oxidation (↓)	(*241*)
beef hot dog	clove oil (5 mL/l)	*L monocytogenes* (1.15−1.71LR)		(*73*)
	thyme oil (1 mL/l)	*L. monocytogenes* (0.67−1.05 LR)		
pork bologna	nisin (125 μg/mL)	*L. monocytogenes* (1.5LR)		(*169*)
minced beef	*Capsicum annum* extract	*Salmonella typhimurium* (Minimum lethal concentration, MLC 15 g/kg)		(*199*)
		Pseudomonas aeruginosa (MLC 30 g/kg)		
chicken frankfurter	clove oil (1% v/w)	*L. monocytogenes* (4.5 LR)		(*197*)
cooked beef	grape seed extract (1%)	*Escherichia coli* (1.7 LR)	color (∼), lipid oxidation (↓)	(*200*)
		S. Typhimurium (2.0 LR)		
		L. monocytogenes (0.8 LR)		
		Aeromonas hydrophila (0.4 LR)		

[a] AU: arbitrary units were defined as the reciprocal of the highest two-fold dilution that did not allow the growth of the indicator strain. AC: anthocyanin content. ↑ and ↓ indicate increase and decrease, respectively, while ∼ shows no significant difference. LR: microbial log reduction.

in the cytoplasmic membrane of bacteria, thus disrupting membrane function and preventing outgrowth of spores by inhibiting the swelling process of germination. It is highly active against many of the Gram-positive bacteria and specifically used by the cheese industry to control the growth of *Clostridium* spp. (*166*). Substantial research has evaluated the efficacy of nisin against various pathogens and its use for different food products (*167*−*174*). Nisin has been used to inhibit microbial growth in beef (*173*), sausages (*2*), liquid whole egg (*174*), ground beef (*175*), and poultry (*176*). It has also been reported to reduce initial levels of *Listeria monocytogenes* and suppress subsequent growth in ready-to-eat (RTE) meat products (*177*, *178*). Komitopoulou et al. (*179*) reported that nisin could be used for the effective control of *Alicyclobacillus acidoterrestris* in fruit juices. A nisin level of 6.25 μg/g could inhibit lactic acid bacteria (LAB) growth for over 28 days and for 35 days with 25 μg/g (*180*). The effects of three types of phosphate (used as emulsifiers) on nisin activity in sausage were compared, and LAB growth rate was fastest in samples containing orthophosphate and slowest in sausages containing diphosphate.

Mechanism of Antimicrobial Action. The antimicrobial action of bacteriocins is based on pore formation in the cytoplasmic membrane of the target microorganism. This leads to a loss of small intracellular molecules and ions and a collapse of the proton motive force (*181*). Nisin is less effective on Gram-negative bacteria, as the outer membrane disables the entry of this molecule to the site of action (*50*, *119*, *182*, *183*). The first step

Scheme 2. Structure of Nisin



in the mode of action of nisin is to pass through the cell wall of Gram-positive bacteria. Generally, it is assumed that nisin passes the cell wall by diffusion. However, the Gram-positive cell wall can act as a molecular sieve against nisin depending on its composition, thickness, or hydrophobicity (*184*). The removal of the cell wall from nisin-resistant *Listeria* resulted in the removal of nisin resistance, suggesting that the cell wall plays a role in the differences in susceptibility toward nisin (*185*). The next step of the antimicrobial process of nisin is to associate with the cytoplasmic membrane of the target microorganism. It has been suggested that nisin interacts electrostatically with the negatively charged phosphate groups of surface membrane phospholipids (*173*).

Factors Affecting Antimicrobial Activity. Various factors can impact the antimicrobial efficacy of bacteriocins. These include the emergence of bacteriocin-resistant bacteria, conditions that destabilize the biological activity of proteins such as proteases or oxidation processes, binding to food components such as fat particles or protein surfaces, inactivation by other additives, poor solubility, and uneven distribution in the food matrix and/or pH effects on bacteriocin stability and activity (*137*). The application of bacteriocins in combination with other preservation hurdles has been proposed to reduce the selection for resistance to bacteriocins in target strains and/or to extend its inhibitory activity to Gram-negative species (*182*). Interactions between bacteriocin and the food matrix may result in a decrease in the efficacy of the bacteriocin. The combination of bacteriocins with other minimal or nonthermal preservation technologies may prove useful for practical applications. This approach is of value for the control of Gram-negative bacteria as their outer membrane acts as an efficient barrier against hydrophobic solutes and macromolecules, such as bacteriocins (*119*).

QUANTIFICATION OF THE MINIMUM AND NONINHIBITORY CONCENTRATION

The use of antimicrobials as preservatives in food systems can be constrained when effective antimicrobial doses exceed organoleptic acceptable levels (especially for essential oils) or when they are added to complex food systems. Two specific concentrations appear to be of interest, i.e., the noninhibitory concentration, NIC, the concentration above which the inhibitor begins to have a negative effect on growth, and the minimum inhibitory concentration, MIC, which marks the concentration above which no growth is observed by comparison with the control (*186*). Therefore, these concentrations are quantified with the aim of defining the boundaries of sensory acceptability and antimicrobial efficacy of antimicrobials (*26*). Most of the studies on the calculation of MIC and NIC are semiquantitative, while quantitative approaches have been mainly applied on studies concerning the antimicrobial activity of plant origin antimicrobial agents, i.e., essential oils and their components.

The MIC and NIC are dependent on experimental conditions. The influencing conditions include the incubation temperature, organism, and inoculum size, and therefore, they should be reported in studies where MIC and NIC are evaluated (*187*, *188*). In vitro studies for identifying the MIC can be divided into groups such as diffusion, dilutions, impedance, and optical density (or absorbance) methods (see for e.g., refs (*189*−*191*)). Most of these evaluations are based on an end-point approach for evaluating the MIC, i.e., end result in which no growth is obtained for a test level of preservative, into which an inoculum of microbes is added. This kind of approach is considered semiquantitative (*188*).

Lambert and Pearson (*188*) examined the inhibitory activity of single compounds of EOs and developed a fully quantitative approach. This is given by the Lambert−Pearson model (LPM) inspired by a modified Gompertz equation (eq 1) to evaluate the dose−responses of microorganisms against several inhibitors. This modeling approach has already been examined for optical density, O.D. (*187*, *188*), and impedance microbial measurements (*62*).

$$fa = \exp\left[-\left(\frac{x}{P_1}\right)^{P_2}\right] \quad (1)$$

In eq 1, *fa* is the fractional area which is defined as the ratio of inhibited growth to uninhibited growth as measured by the applied method (impedance, optical density, etc.), x is the inhibitor concentration (mg/L), P_1 is the concentration at maximum slope (of a log x vs *fa* plot; see **Figure 1** for a graphical example of this equation), and P_2 is a slope parameter. Observe that *fa* can be measured by using the trapezoidal rule under the O. D. (or other microbial measurements)/time curves and then taking the ratio of the test area to that of the control (*187*). Therefore, the range of *fa* will be between 0 and 1 (**Figure 1**). The routine, trapz, provided by Matlab is an example of a software package that can be used for performing a trapezoidal numerical integration.

The MIC (eq 2) and the NIC (eq 3) can then be calculated as the intercept of the concentration axis to the tangent at the maximum gradient of the *fa*/log concentration curve and the intercept of the tangent at the maximum gradient of the *fa*/log concentration curve to the $fa = 1$ contour.

$$MIC = P_1 \cdot \exp\left(\frac{1}{P_2}\right) \quad (2)$$

$$NIC = P_1 \cdot \exp\left(\frac{1-e}{P_2}\right) \quad (3)$$

Guillier et al. (*192*) developed another approach for evaluating the MIC based on the use of growth rate models. After estimation of the maximum specific growth rates (μ_{max}) from optical density

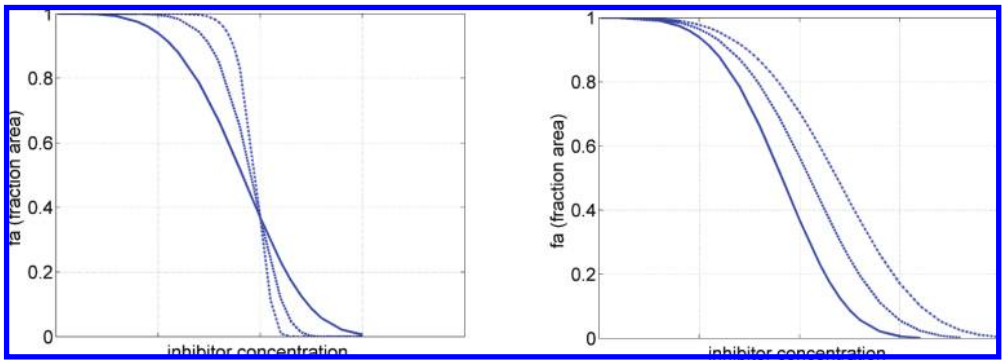


Figure 1. Hypothetical inhibition profile as can be described by eq 1 for increasing values of P_2 and constant P_1 (**left panel**) and increasing values of P_1 and constant P_2 (**right panel**). Inhibitor concentration is expressed on a logarithmic scale.

growth kinetics by a modified Gompertz model, they assessed the antimicrobial concentration dependence on μ_{max} (eq 4).

$$\sqrt{\mu_{max}} = \sqrt{\mu_{max}(c = 0)\cdot f(c)} \quad (4)$$

$f(c)$ can be described either as eq 5, i.e., the SR_μ model, or as eq 6, i.e., the LP_μ model.

$$f(c) = \left(1 - \frac{c}{MIC}\right)^{\beta}, c < MIC \text{ or } 0, c \geq MIC \quad (5)$$

$$f(c) = \exp\left[-\left(\frac{c}{MIC/\exp\left(\frac{\ln(NIC/MIC)}{-e}\right)}\right)^{-e/(\ln(NIC/MIC))}\right] \quad (6)$$

$\mu_{max}(c = 0)$ is the growth rate in the absence of the antimicrobial ($c = 0$) and β a shape parameter representing the sensitivity of the microorganism to an antimicrobial in eq 5. These two approaches appeared to give equivalent results. Observe that for estimating the parameters of MIC, NIC, and $\mu_{max}(c = 0)$ of eq 6, a regression is performed for the data that relate the maximum specific growth rates (μ_{max}) with the concentration of the inhibitor.

Lambert et al. (*26*) argued that the majority of antimicrobial activity could be attributed to two components acting independently. Therefore, they also suggested another expression for a mixture of two inhibitors that could be extended in case there are more inhibitors as presented in eq 7:

$$fa_{x_i,\ldots,x_k} = \exp\left\{-\left[\left(\frac{x_i}{C_{i,1}}\right)^{C_{i,2}} + \ldots + \left(\frac{x_k}{C_{k,1}}\right)^{C_{k,2}}\right]^{C_Q}\right\} \quad (7)$$

where parameters $C_{i,1}$ are the concentrations of the x_i inhibitors at the maximum slope. The main difference is that the current expression takes into account interactions between the antimicrobials, which means that it could be considered for any additive, antagonistic, and synergistic activity between the studied inhibitors. For an example in which a mixture of two antimicrobials is studied reference is made to Lambert and Lambert (*187*). In that case, the MIC of any of the x_i antimicrobials is then given by eq 8.

$$MIC = C_{i,1}\cdot\exp\left(\frac{1}{C_{i,2}+C_Q}\right) \quad (8)$$

Another interesting quantitative approach for evaluating the bactericidal effect of different agents has been suggested by Lui et al. (*193*). This is based on a concentration killing curve approach and the estimation of the so-called median bactericidal concentration and bactericidal intensity. The developed method is based on the correlation (by the use of a sigmoidal curve with an inflection point) of the population size (number CFU per plate) with respect to the concentration of the agent. This approach has been applied for quantifying the bactericidal potency of antibiotics against *E. coli* and might have to be further investigated for different antimicrobials. Similar to the discussed approaches, novel modeling methods for quantitatively expressing the effect of antimicrobials through MIC and NIC values can be developed by knowledge coming from predictive microbiology. An overview of representative cases for different modeling expressions tackling the effect of both chemical and natural inhibitory compounds can be found in Devlieghere et al. (*194*).

Accurate quantitative evaluations of MIC and NIC are important for designing effective preservation methods that are based on the use of the discussed antimicrobials. These quantitative methods can be exploited to give insight to optimal concentrations or combinations for real food systems by direct comparison of the antimicrobial efficacy of different antimicrobials, their individual or combined components, or their mixtures, and for efficient design of preservation for food products based on the principles of hurdle technology. These approaches have not received much attention for evaluating the MIC or the minimum bactericidal concentration of the antimicrobials of animal and microbial origin, but their potential is evident.

APPLICATIONS OF NATURAL ANTIMICROBIALS IN FOOD

The extrapolation of results obtained from in vitro experiments with laboratory media to food products is not straightforward as foods are complex, multicomponent systems consisting of different interconnecting microenvironments. Though there is vast potential for natural antimicrobial agents in food preservation, most of the literature presents inactivation data from model foods or laboratory media. **Table 1** reports inactivation studies in real food systems. The level of natural preservatives required for sufficient efficacy in food products in comparison with laboratory media may be considerably higher, which may negatively impact the organoleptic properties of food.

Monoacylglycerols have increased the shelf life of various foods including soy sauce, miso, sausages, cakes, and noodles (*36*). The lauric acid ester of monoacylglycerol has reported antimicrobial potential in seafood salads and various flesh foods including deboned chicken meat, minced fish, refrigerated beef roasts, and frankfurter slurries (*126*, *195*). Hao et al. (*196*) studied the efficacy of a range of plant extracts for inhibition of *A. hydrophila* and *L. monocytogenes* in refrigerated cooked poultry and found that eugenol reduced pathogen counts by 4 $\log_{10}$ cfu/g over a 14 day storage trial. Similarly, 1−2% w/w clove oil inhibited the growth of a range of *Listeria* spp. in chicken frankfurters over 2 weeks at 5 °C (*197*). Conversely, Shekarforoush

et al. (*198*) found that EOs of oregano and nutmeg were effective against *E. coli* O157:H7 in a broth system but had no effect in ready-to-cook chicken. Careaga et al. (*199*) recorded that 1.5 mL/100 g of capsicum extract was sufficient to prevent the growth of *S. typhimurium* in raw beef but that 3 mL/100 g was required for a bactericidal effect against *P. aeriginosa*. Ahn et al. (*200*) also found a range of plant extracts to be useful for reduction of pathogens associated with cooked beef and quality maintenance; however, Uhart et al. (*201*) concluded that when in direct contact, spices inactivated *S. typhimurium* DT104 but that the activity decreased considerably when added to a complex food system such as ground beef. Gutierrez et al. (*74*, *75*) concluded that plant essential oils are more effective against food-borne pathogens and spoilage bacteria when applied to ready-to-use foods containing a high protein level at acidic pH as well as lower levels of fats or carbohydrates and moderate levels of simple sugars. The success of plant derived antimicrobials when applied to fruit and vegetable products is also documented in the literature. Karapinar et al. (*202*) recommended unripe grape juice as an alternative antimicrobial agent for enhancing the safety of salad vegetables, and Martinez-Romero et al. (*203*) suggested that carvacrol could be applied as a novel tool for the control of fungal decay on grapes. Although Valero and Frances (*204*) found that low concentrations of carvacrol, cinnamaldehyde, or thymol had a clear antibacterial effect against *B. cereus* in carrot broth, cinnamaldehyde retained a significant activity at storage temperatures of 12 °C. Gutierrez et al. (*205*) found that the efficacy of oregano EO was comparable with chlorine as a decontamination treatment for ready-to-eat carrots. Use of this essential oil contributed to the acceptability of sensory quality and appreciation. A novel application of plant extracts is for the production of chocolate; Kotzekidou et al. (*206*) reported enhanced inhibitory effects of plant extracts against an *E. coli* cocktail at 20 °C.

Antimicrobials from microbial sources, especially nisin, find application in a number of foods such as milk, orange juice (*207*), and tomato juice (*208*), and for increasing the shelf life of chicken meat without altering sensory properties of the product (*209*). The efficacy of enterocin AS-48 for inhibition of *B. cereus* in rice and *S. aureus* in vegetable sauces was investigated (*210*, *211*) with bacteriocin levels in the range of 20−35 μg/mL and 80 μg/mL, respectively.

Investigation of the antimicrobial properties of preservatives from animal sources and their possible potential in food application is still in its infancy, with few published studies available as described above. A common conclusion that could be drawn from these studies is the fact that the significant potential of antimicrobials from animal sources is not being exploited.

Some other applications in foods that got attention in previous years are the use of bioactive packaging technologies. These systems can be applied for all of the discussed antimicrobials, i.e., plant, animal, and microbial origin agents either by adding a sachet (or possibly by encapsulating the agents (*212*)) into the package, dispersing bioactive agents in the packaging, coating bioactive agents on the surface of the packaging material, or utilizing antimicrobial macromolecules with film-forming properties or edible matrixes (*213*, *214*). Film-coating applications have been reported for meat, fish, poultry, bread, cheese, fruits, and vegetables (*215*).

USE OF NATURAL ANTIMICROBIALS IN THE MULTIPLE-HURDLE CONCEPT

Investigations based on combinations of natural antimicrobials with other nonthermal processing technologies within the multiple-hurdle concept are warranted to counteract any potential organoleptic or textural effects on food products as well as optimizing microbial inactivation. The preservative action of bacteriocins alone in a food system is unlikely to ensure comprehensive safety. This is of particular significance with regard to Gram-negative pathogenic bacteria that are protected from the antimicrobial action of bacteriocins by the presence of an outer membrane. When the outer membrane is disrupted by agents such as the food grade chelating agent ethylene diamine tetraacetate (EDTA), which acts by binding to Mg^{2+} ions in lipopolysaccharide, the outer membrane of Gram-negative bacteria are rendered sensitive to the antimicrobial action of bacteriocins (*181*). Potential synergistic effects may be found with other chemical or physical inactivation technologies including dense phase carbon dioxide, ultrasound, pulsed-electric field, high pressure, and ozone treatment. As a consequence of applying these nonthermal methods, bacterial cell membranes can weaken or become susceptible to additional antimicrobial agents such as bacteriocins, causing lethality. The use of bacteriocins in combination with organic acids or other antimicrobials can similarly result in enhanced inactivation (*216*). Studies reporting the effective use of nisin against Gram-negative organisms and fungi are those in which nisin was used in combination with traditional food preservatives such as organic acids and chelating agents (*217*). Rajkovic et al. (*218*) found that the activity of nisin combined with carvacrol was enhanced in a potato puree by comparison with BHI broth and that more obvious effects against *B. cereus* and *B. circulans* were observed at higher temperatures. The application of bacteriocins in combination with treatments that could enhance their effectiveness in foods requires investigation. Examples of the synergistic effects that can be obtained using mild traditional preservation techniques in conjunction with novel food processing technologies are better studied in vitro but require further investigation in food products to ensure successful practical application. The antibacterial activity of inhibitory compounds, such as nisin, enterocin, monolaurin, and the lactoperoxidase system (LPS), can be enhanced if applied in combination (*219*−*221*), with chelating agents (*182*, *222*, *223*) or with preservative treatments such as high hydrostatic pressure, pulsed electric field, low pH, or freeze/thaw cycles (*224*−*228*). The combination of plant EOs with modified atmosphere packaging for control of spoilage species was reported by Skandamis and Nychas (*229*) and Matan et al., (*230*). Seydim and Sarikus (*231*) also investigated the use of EOs in an active packaging system based on an edible whey protein film and concluded that oregano was the most effective EO against a range of food pathogens. Allyl isothiocyanate was successfully applied to chopped, refrigerated, nitrogen packed beef for the control of *E. coli* at levels in excess of 1000 ppm.

Conclusions and Future Trends. Interest in natural antimicrobials has expanded in recent years in response to consumer demand for greener additives. During the last two decades, natural preservatives have been investigated for practical applications. These technologies have been shown to inactivate microorganisms and enzymes without significant adverse effects on organoleptic or nutritional properties. Reported studies have demonstrated that natural antimicrobial agents described in this review may offer unique advantages for food processing. In addition to improving the shelf life and safety of foods, natural antimicrobial agents may allow novel food products with enhanced quality and nutritional properties to be introduced to the market.

The applications of natural antimicrobial agents are likely to grow steadily in the future because of greater consumer demands for minimally processed foods and those containing naturally derived preservation ingredients. More complex considerations arise for combinations of technologies, particularly with respect

to optimization of practical applications. Intelligent selection of appropriate systems based on detailed, sequential studies and quantitative approaches to evaluate the efficiency of antimicrobials is necessary. The impact of product formulation, extrinsic storage parameters, and intrinsic product parameters on the efficacy of novel applications of combined nonthermal systems requires further study.

ABBREVIATIONS USED

Abu, amino butyric acid; Ala, alanine; asn, asparagine; Dha, dehydroalanine; Dhb, dehydrobutyrine (β-methyldehydroalanine); Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine ; Lys, lysine ; Met, methyonine ; Pro, proline; Ser, serine; Val, valine.

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