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J.F. Ayala-Zavala Centro de Investigación en Alimentación y Desarrollo, Hermosillo, Mexico

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Preface

The protection of microbiological safety and keeping quality of foods for human consumption is essential for the assurance of a nutritious and sustainable food supply. Excepting only those foods processed so as to render them microbiologically sterile, thereby free of all viable microorganisms, following final packaging, any food may be subject to loss of microbiological safety and/or microbiologically driven spoilage if requisite conditions for microbial growth and metabolism of food components are met for contaminating microbes. It is the inhibition of this microbial growth and consumption/decomposition of constituents of the food (i.e., amino acids from proteins, utilizable carbohydrates, lipids, etc.) that provides cause for food processors and food microbiologists to investigate the application of differing antimicrobial compounds in various foods. Food antimicrobials are chemicals that exert a growth-inhibiting effect, and in some cases may possess microbicidal activity, though this is not typically observed in food products. As opposed to the host of antibiotics that are utilized in the United States and throughout the world to combat infectious pathogens in food-yielding animals, this text will explore the use of various types of antimicrobials that are approved by relevant food manufacture/process-regulating agencies (e.g., U.S. Food and Drug Administration, U.S. Department of Agriculture-Food Safety and Inspection Service) for direct or indirect application in the manufacture of foods for human consumption.

In recent decades, there has been substantial output of scientific research investigating the efficacy and utility of a host of chemical preservatives for the preservation of differing foods. In addition to continued interests in compounds such as weak organic acids (e.g., lactic acid, citric acid, acetic acid), various surfactant-type and polymeric antimicrobials (e.g., poly-L-lysine, lauric arginate ester), oxidizing sanitizers (e.g., chlorine compounds, ozone, peroxides), and others, a great deal of focus has been directed at the evaluation of different types of naturally occurring compounds with antimicrobial activity. In particular, much research has focused on the characterization of plant-derived antimicrobials for inhibiting the growth of both foodborne pathogen and spoilage microbe alike. Numerous plant extracts, fractions, and specific constituents from their essential oils have been reported in the scientific literature, adding to the body of knowledge surrounding the utility of such systems for the inhibition of microbial organisms' growth in foods, whether pathogenic or spoilage. Great strides have also been made in determining the genetics and expression regulation of bacterially produced and secreted antimicrobial polypeptides, most notably the bacteriocins produced by various Gram-positive organisms, and to a lesser extent, the colicins by some

Gram-negative microbes. Interest in the use of microorganisms, or the fermentate of some microbes, as pathogen or spoiler antagonists (i.e., biopreservation) has been renewed in recent years as well; in the United States as well as other industrialized nations, multiple antimicrobial products have gained regulatory approval and have been marketed to food processors, consisting of homofermentative and heterofermentative microbes exerting pathogen-control effects (e.g., lactobacilli, pediococci, lactococci), or bacteriophages that infect and lyse a sensitive microorganism, releasing phage particles that may infect and kill nearby cells of the same microbe (e.g., phages specifically infective to pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* serovars).

These and other types of research have been conducted against an ever-evolving backdrop of food quality, processing and labeling, and safety concerns. Whereas many traditional-type antimicrobials were previously investigated and approved for their utility in preventing loss of product shelf life/keeping quality, the focus of much research today centers on the use of food antimicrobials for the inhibition or inactivation of microbial foodborne pathogens. One significant driver of this concern has been the identification and characterization of the development of antibiotic-resistant and multidrug-resistant pathogens, with the subsequent inability of many relied-upon therapeutic agents to combat and relieve human disease by these microbes should they be transmitted to consumers via contaminated food(s) consumption. Chemical preservatives are generally thought to not exert their inhibitory action by the same mechanisms as some antibiotic agents, circumventing the mechanisms of drug resistance by the pathogen. Nonetheless, microbial adaptation to chemical food preservatives, such as the organic acids, has been described as transient in nature under certain conditions of exposure, and so is not transmitted vertically through multiple generations of the microorganism(s) of concern.

In addition, food processors have recognized increasing consumer desire for "clean" labeling of foods, another factor that has encouraged greater research into natural food antimicrobials. The desire of consumers to have less extensively processed foods has resulted in a simultaneous need for greater process control while effectively removing from use some preservatives that would provide for shelf-life preservation, due to the perception that such compounds are unnatural and thus are undesirable. In the United States, in particular, this shift has occurred alongside the development of several new food process regulations (e.g., mandatory implementation of HACCP for some food commodities, control of *L. monocytogenes* in postlethality-exposed meat/poultry) and the recent passage of the Food Safety Modernization Act (21 U.S.C. 2201 *et seq.*). These and other factors are likely to continue to spur new research into antimicrobials, as processors seek data validating the utility of their process interventions for food safety control within the frameworks of HACCP and Current Good Manufacturing Practices.

The purpose of this text is not to attempt to replace previously published texts that have summarized the state of the knowledge at their publication. Further, there are numerous quality texts available to the interested reader in addition to this one. This text seeks primarily to summarize our knowledge of the usefulness, efficacy, applications, and limitations to use of natural food antimicrobial preservatives, focusing at

times on antimicrobials that may not be widely approved for use throughout various food types or countries, and at others on compounds that have received substantial attention and have proven utility in certain classes of food products (e.g., beverages). Chapters have been authored by topic experts from across the globe, bringing an international appeal and character to the text. The nature of the research reviewed within chapters ranges from the more fundamental to highly translational work, with opportunity for adaptation by the food process industry. The editor thanks the authors of the text chapters for their contributions, responsiveness to editorial comment and cooperativity in assisting the development, preparation, and publication of this text. On behalf of the authors, the editor hopes that readers will find the text a useful and functional resource for citation of completed research in the field of food antimicrobial use in the preservation of food safety and quality, and that it will encourage students and developing scientists to enter into scientific research that seeks to improve and protect the microbiological safety and quality of foods for human consumption worldwide.

T. Matthew Taylor
Texas A&M University, College Station, TX, USA

The use of natural antimicrobials in food: an overview

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P.M. Davidson, H. Bozkurt Cekmer, E.A. Monu, C. Techathuvanan
University of Tennessee, Knoxville, TN, USA

1.1 Introduction

Microorganisms are present throughout the food supply and can contaminate food in various ways, including at the farm level through irrigation water, field workers, insects, and fecal contamination by wild animals, as well as postharvest sources, such as handling by workers, transport vehicles, and processing equipment; wash water; and cross-contamination from other foods. These microorganisms pose two major problems to the food supply: the risk to human health from foodborne illness and the economic losses associated with food loss because of spoilage. Scallan, Griffin, Angulo, Tauxe, and Hoekstra (2011) and Scallan, Hoekstra, et al. (2011) estimated that approximately 48 million cases of foodborne illness occur in the United States annually, 128,000 of which result in hospitalizations and 3000 deaths. It also has been estimated that 31% of the available food supply at the retail and consumer levels in 2010 was not consumed, which is equivalent to an economic loss of approximately $161.6 billion (Buzby, Wells, & Hyman, 2014). Part of this loss is due to food being discarded because of spoilage either in the retail market before it can be purchased by consumers or in the homes of consumers (Kantor, Lipton, Manchester, & Oliveira, 1997). Economic loss can also be incurred from foodborne illness, not only because of hospital costs and lost work, but also as a result of recalls. In 2008, an outbreak of *Listeria monocytogenes* from deli meat in Canada that caused 20 deaths cost a company more than $50 million in recalls, restructuring costs, and market losses as well as $27 million in settlements (Greenberg & Elliott, 2009).

Antimicrobials are chemical compounds that are naturally present in or added to foods, food packaging, food contact surfaces, or food processing environments to inhibit microbial growth or kill microorganisms. The primary functions of antimicrobial food preservatives are to inhibit or inactivate pathogenic and spoilage microorganisms (Davidson & Zivanovic, 2003). The use of antimicrobials is useful to reduce food losses caused by microbiological spoilage and to assist in ensuring microbiological safety. According to Davidson, Critzer, and Taylor (2013), an ideal naturally occurring antimicrobial should (1) be effective at low concentrations in its natural form, (2) be economical, (3) cause no sensory changes to the product, (4) inhibit a wide range of pathogenic and spoilage microorganisms, and (5) be nontoxic.

Although antimicrobial preservatives are already available for use in the food industry, many producers are searching for natural antimicrobial alternatives. There are several reasons for this trend. The most important is that the traditional

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regulatory-approved antimicrobials have very limited capabilities when it comes to controlling spoilage or pathogenic microorganisms. Many are organic acids and function well only at a low pH, whereas often the major problems with food safety are in foods with a near neutral pH. Because of the generally unfounded fears that synthetic antimicrobials pose possible toxicological problems, there is also a growing demand by consumers for foods with ingredients that contain fewer synthetic additives (David, Steenson, & Davidson, 2013; Davidson & Zivanovic, 2003; Sofos, Beuchat, Davidson, & Johnson, 1998). This has led food companies to seek "green" or "clean" labels. Recommendations for health may also contribute. For example, agencies such as the World Health Organization are advising consumers to reduce their intake of salt to decrease the risk of cardiovascular disease, but salt is commonly used in the preservation of a wide variety of foods (World Health Organization, 2002). The reduction of salt in processed foods could lead to the need for additional preservatives to ensure the safety and maintain the shelf life of products.

There likely will be few, if any, new synthetic antimicrobials coming onto the market. This is, in part, because of the strict requirements of international regulatory agencies to gain approval for novel direct food additives (Davidson & Zivanovic, 2003). For example, the European Food Safety Authority requires rigorous toxicological testing of these antimicrobials, including metabolism and toxicokinetics, subchronic toxicity, reproductive and developmental toxicity, and genotoxicity (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2012). These batteries of tests, including *in vitro* and *in vivo* tests in animals and humans, can take years and a large amount of money before they can be completed to obtain approval, making the pursuit of these antimicrobials unprofitable (Davidson & Zivanovic, 2003).

Another appeal of natural antimicrobials is that several of them, particularly plant-based extracts, have potential health benefits for humans. Mustard, for example, contains isothiocyanates, which have wide-spectrum antimicrobial activity and have been shown to have potential chemopreventive activity, along with several other compounds found in mustard (Vig, Rampal, Thind, & Arora, 2009). Garlic has been used medicinally for thousands of years, and we now know that the organosulfur compounds (most notably allicin) have antimicrobial activity against a variety of bacteria and fungi, as well as chemopreventive and antioxidant activity, reducing the risk of cardiovascular disease and improving immune function (Benkeblia, 2004; Lau, 2006; Rahman, 2007). Other plant essential oils, such as antioxidants, also have health benefits to help combat the degenerative diseases of aging by protecting low-density lipoprotein cholesterol from oxidation, inhibiting cyclooxygenase and lipoxygenase enzymes, and inhibiting lipid peroxidation. Several also have antiviral or antitumor activity (Craig, 1999). These added health benefits may be indirect rather than through the consumption of the plant extract itself, such as in the case of grape seed and rosemary extracts. Gibis and Weiss (2012) found that the addition of grape seed and rosemary extracts to marinades for ground beef patties reduced the formation of possibly carcinogenic heterocyclic amines. The same study also indicated that the extracts exhibited antioxidant activity in the beef patties, showing that, in addition to health benefits, there may also be positive effects on food quality other than the inhibition

of spoilage microorganisms. Green tea and grape seed extracts can also reduce lipid oxidation in beef, chicken, and turkey (Perumalla & Hettiarachchy, 2011).

Ironically, while consumers are very clear about their desire for more natural food additives, there seems to be a lack of knowledge that several of the antimicrobial preservatives currently used by the food industry are found in nature. For example, sorbic acid is a food antimicrobial used primarily to inhibit yeasts and mold, and although the commercial form used today is created through chemical synthesis, it was originally discovered by A.W. von Hoffman in 1859 from the oil of unripened rowanberries (Stopforth, Sofos, & Busta, 2005). Although this compound is found in nature and was originally isolated from plants, the fact that it is now chemically synthesized means that it is subject to regulatory approval requirements for use in food and use of scientific nomenclatures required by laws and can no longer contribute to a "green label." The situation is similar for several other organic acids used as food antimicrobials, including acetic acid (found in vinegar), benzoic acid (found in cranberries), lactic acid (produced in a variety of fermented foods by lactic acid bacteria), and propionic acid, which is produced by *Propionibacterium freudenreichii* ssp. *shermani* in Swiss cheese, giving the characteristic holes ("eyes") and nutty flavor (Fröhlich-Wyder & Bachmann, 2004).

1.2 Types of natural antimicrobials: animal sources

Natural antimicrobials have been identified and isolated from animal, plant, and microbial sources. Following are brief descriptions of some of the more studied natural antimicrobials. The subsequent chapters discuss most of these compounds in depth.

1.2.1 Lysozyme

Lysozyme is a widely occurring enzyme that hydrolyzes the β-1,4-glycosidic bond between C1 of *N*-acetylmuramic acid and C4 of *N*-acetylglucosamine in peptidoglycans. The enzyme is affirmed as generally regarded as safe (GRAS) and approved for direct addition to foods in the United States (Davidson, Taylor, & Schmidt, 2012). Although often isolated from chicken eggs, lysozyme is widespread in nature and found in many biological sources including certain vegetables, insects, plants, and fungi. Lysozyme is also present in human colostrum and mammalian tissues and fluids such as milk, saliva, mucus, blood, and tears (Johnson & Larson, 2005).

Lysozyme is most active against Gram-positive bacteria when peptidoglycan is accessible, but it is inactive against Gram-negative bacteria because of the presence of the outer membrane. It shows general activity against species of *Bacillus*, *Micrococcus*, and *Clostridium* as well as strain-specific activity against *L. monocytogenes* and *Lactobacillus* spp. (Davidson et al., 2013). The presence of teichoic acids and other materials binding the enzyme causes the variation in susceptibility among Gram-positive bacteria. There are certain Gram-positive bacterial species that have greater proportions of 1,6- or 1,3-glycosidic linkages in the peptidoglycan, which are more resistant than the 1,4 linkage (Davidson et al., 2012). Reduced peptidoglycan content (5−10%) and the presence of a

lipopolysaccharide in the outer membrane that blocks access of the enzyme are primary reasons for the reduced sensitivity to lysozyme by Gram-negative bacteria. Their sensitivity can be increased by chelators (e.g., ethylenediaminetetraacetic acid (EDTA)) that bind Ca^{++} or Mg^{++}, which are essential for maintaining the integrity of the lipopolysaccharide layer (Davidson & Zivanovic, 2003). Lysozyme is also inhibitory to other microorganisms including fungi such as *Aspergillus*, *Candida*, *Fusarium*, *Sporothrix*, *Paecilomyces*, *Penicillium*, and *Saccharomyces*.

1.2.2 Lactoperoxidase system

Lactoperoxidase, an enzyme found in the secretions of mammalian species (raw milk, saliva, colostrum, etc.), catalyzes the oxidation of thiocyanate (SCN^-), iodide, or bromide by hydrogen peroxide to generate reactive products such as hypothiocynate ($OSCN^-$) and hypothiocyanous acid, which have antimicrobial activity (Stopforth, Skandamis, Davidson, & Sofos, 2005). Microbial inhibition or inactivation is a result of oxidation of sulfhydryl groups in enzymes and other key proteins, which leads to structural damage to the cytoplasmic membrane and leakage of potassium ions, amino acids, and peptides into the surrounding medium. The lactoperoxidase system causes more extensive damage to the inner membrane of Gram-negative bacteria (e.g., *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella*, *Yersinia enterocolitica*) than Gram-positive bacteria (e.g., *L. monocytogenes, Staphylococcus aureus*, and *Bacillus cereus*), especially at low temperatures (Kussendrager & van Hooijdonk, 2000). Gram-negative catalase-positive bacteria are not only inhibited but also killed depending on the environmental conditions, whereas Gram-positive catalase-positive bacteria are generally inhibited but not necessarily killed by the lactoperoxidase system (Stopforth, Skandamis, et al., 2005). The antifungal activity of lactoperoxidase also has been demonstrated against *Aspergillus*, *Byssochlamys fulva*, *Candida*, *Mucor*, and *Rhodotorula* (Davidson et al., 2013).

Lactoperoxidase can play an active role in the keeping quality of raw and pasteurized milk (Babu, Varshney, & Sogi, 2004; Barrett, Grandison, & Lewis, 1999; Parry-Hanson, Jooste, & Buys, 2009) and has exhibited inhibitory activity against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella*, and *Y. enterocolitica* in products such as beef, vegetable juices, milk, and liquid whole egg (Elliot, Mclay, Kennedy, & Simmonds, 2004).

1.2.3 Lactoferrin

Lactoferrin is a member of the transferrin family of iron-binding proteins found in milk, colostrum, other physiological fluids, and polymorphonuclear leukocytes (Farnaud & Evans, 2003). The proposed antimicrobial mechanism of lactoferrin is through chelation of iron and possibly through direct interaction between the protein and target microorganism (Davidson et al., 2012). Lactoferrin is inhibitory to many foodborne microorganisms, including micrococci, *B. subtilis*, *Bacillus stearothermophilus*, *Carnobacterium viridans*, *L. monocytogenes*, *E. coli*, *E. coli* O157:H7, *Klebsiella* spp., and *Salmonella* Enteritidis (Davidson et al., 2013). However, microorganisms with low iron needs,

such as lactic acid bacteria, are not sensitive to lactoferrin (Pandey, Bringel, & Meyer, 1994).

Enzyme-hydrolyzed lactoferrin is an antimicrobial peptide obtained from the pepsin N-terminal cleavage of lactoferrin (Arseneault, BéDard, Boulet-Audet, & PéZolet, 2010). The active peptides responsible for the antimicrobial activity of the lactoferrin hydrolysate were subsequently identified and sequenced by and Bellamy, Takase, Wakabayashi, Kawase, and Tomita (1992) and were named lactoferricin H (from human milk) and lactoferricin B (from bovine milk). Hydrolyzed lactoferrin was found to be 9 to 25 times more effective than lactoferrin against several Gram-negative and Gram-positive microorganisms. In addition, the hydrolysate was found to be bactericidal (Tomita et al., 1991), whereas lactoferrin is only bacteriostatic. The antimicrobial spectrum of lactoferricin is wide (Bellamy, Takase, Wakabayashi, Kawase, & Tomita, 1992; Branen & Davidson, 2000; Jones, Smart, Bloomberg, Burgess, & Millar, 1994; Shin et al., 1998), inhibiting both Gram-positive and Gram-negative bacteria (including several foodborne pathogens), yeasts, molds, and even parasites (Tanaka et al., 1995). The minimum inhibitory concentrations (MICs) of lactoferricin are low, ranging from 0.5 to 500 mg/ml for both Gram-positive and Gram-negative bacteria (Bellamy et al., 1992; Jones et al., 1994). Lactoferricin also has been shown to be active over a wide pH range (Bellamy et al., 1992) and is resistant to heat. For example, lactoferricin hydrolysate maintained activity after autoclaving at 121 °C for 15 min (Tomita et al., 1991). However, the activity of lactoferricin decreased in the presence of cations and salts (Bellamy et al., 1992; Branen & Davidson, 2000; Jones et al., 1994); therefore, despite its strong antimicrobial capabilities, it may not be useful as an antimicrobial in foods.

1.2.4 Chitosan

Chitosan is a natural component derived from alkaline-hydrolyzed shellfish chitin that consists of polymeric 1,4-linked 2-amino-2-deoxy-D-glucose and comprises a series of polymers with different ratios of glucosamine and *N*-acetylglucosamine. Chitosan inhibits growth of foodborne molds, yeasts, and bacteria including *Aspergillus flavus*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Mucor racemosus*, *Byssochlamys* spp., *Botryis cinerea*, *Rhizopus stolonifer*, *Salmonella*, *S. aureus*, *Y. enterocolitica*, *L. monocytogenes*, and *Lactobacillus fructivorans*. MICs for both bacteria and yeasts vary widely, from 0.01% to 5.0%, depending on the molecular weight of the polymer, degree of acetylation, pH, temperature, and presence of interfering compounds such as proteins and lipids (Davidson & Zivanovic, 2003). Even though the mode of action is not completely understood, the two suggested mechanisms are (1) the binding of cationic chitosan to sialic acid in phospholipids, consequently restraining the movement of microbiological substances (Chatterjee, Adhya, Guha, & Chatterjee, 2005) and (2) penetration of oligomeric chitosan into the cells of microorganisms and prevention of the growth of cells by preventing the transformation of DNA into RNA (Sashiwa & Aiba, 2004).

In addition to its antimicrobial properties, chitosan can also form gels, films, fibers, sponges, beads, and nanoparticles because of its high molecular weight and solubility

in acidic aqueous solutions (Zivanovic, Chi, & Draughon, 2005). Chitosan films have a potential to be used as a packaging material because of its excellent oxygen and carbon dioxide barrier properties and could be combined with other antimicrobials to enhance inhibition of microorganisms and improve applicability (Davidson et al., 2013; Jridi et al., 2014).

1.3 Types of natural antimicrobials: plant sources

1.3.1 Spices and their essential oils

Essential oils isolated from widely used spices and their plant sources contain compounds known for their antimicrobial activity (Dorman & Deans, 2000). Oils containing spices that have shown greatest activity against microorganisms are cloves, cinnamon, oregano, and thyme, and their primary components are eugenol, cinnamaldehyde, carvacrol, and thymol, respectively (Deans, Noble, Hiltunen, Wuryani, & Penzes, 1995; Holley & Patel, 2005; Pyrgotou, Giatrakou, Ntzimani, & Savvaidis, 2010). Other essential oils or extracts from melissa, basil, bay, cilantro (coriander), citrus, cumin, dill, fingerroot, laurel, lemongrass, marjoram, nutmeg, rosemary, sage, savory, tea tree oil, vervain, and yerba mate have demonstrated moderate to high activity against selected microorganisms in some studies (Davidson et al., 2013).

Eugenol [2-methoxy-4-(2-propenyl)-phenol] is the major antimicrobial in clove bud oil, comprising approximately 70−90% of the oil. The remainder of the oil contains compounds such as eugenol acetate (0−15%) and β-caryophyllene (5−15%) (Alma, Ertas, Nitz, & Kollmannsberger, 2007; Deans et al., 1995; Holley & Patel, 2005; Pino, Marbot, Agüero, & Fuentes, 2001). The antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) is attributed to cinnamic aldehyde (3-phenyl-2-propenal), l-linalool (2,6-dimethyl, 2-7-octadien-6-ol), *p*-cymene, and eugenol. Cinnamon contains 0.5−1.0% volatile oil, which contains 65−75% cinnamic aldehyde and 8% eugenol (Vigil, Palaou, & Alzamora, 2005). Cinnamon and cinnamic aldehyde are the most effective among the spice essential oils and their components, respectively. The antimicrobial activity of thyme oil is most readily attributed to thymol [5-methyl-2-(1-methyl)-phenol], which is generally present in concentrations around 45% (Bagamboula, Uyttendaele, & Debevere, 2003). However, thyme oil may also contain large quantities of carvacrol, which can range from 33% in leaf oils to about 61% in stem oil, depending on geographic location, time of harvest, or extraction method of the oil (Belaqziz et al., 2013; Ultee, Bennik, & Moezelaar, 2002). Oregano oil is one of the most common sources of carvacrol, which makes up 60−70% of the oil. In addition to carvacrol, thymol, pinene, and *p*-cymene are other major antimicrobial components of oregano (Vigil et al., 2005).

Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, whereas less is known about their action on yeast and molds. Gram-negative bacteria are generally less susceptible to essential oils than Gram-positive bacteria (Trombetta et al., 2005). The outer membrane of Gram-negative bacteria contains hydrophilic lipopolysaccharides, which create a barrier against macromolecules and hydrophobic compounds, providing Gram-negative

bacteria with higher tolerance toward hydrophobic antimicrobial compounds like those found in essential oils (Nikaido, 1994, 2003). Most essential oil constituents have multiple targets. It is therefore difficult to predict how susceptible a microorganism is and why susceptibility varies from strain to strain. Predictions about the mode of action of crude essential oils require thorough investigation of their constituents' target sites, mode of action, and interactions with the surrounding environment (Hyldgaard, Mygind, & Meyer, 2012). Increased membrane permeability, dissipation of proton motive force, inhibition of adenosine triphosphate synthesis, and enzyme inhibition also have been suggested as antimicrobial mechanisms of action for essential oils and their components (Davidson et al., 2013).

1.3.2 Cranberries and other berries

Berry fruits are rich sources of bioactive compounds, such as polyphenolics and organic acids, which have antimicrobial activities against human pathogens. Cranberry, cloudberry, raspberry, strawberry, and bilberry possess clear antimicrobial effects against *Helicobacter pylori*, *L. monocytogenes*, *Salmonella*, *S. aureus*, *E. coli*, and *Campylobacter* spp. (Côté et al., 2011).

Inhibition of the growth of microorganisms observed with berries is linked to low pH, but it also was hypothesized that bioactive compounds, such as phenolics and organic acids, may contribute to the observed antimicrobial actions (Wu, Qiu, Bushway, & Harper, 2008). The antimicrobial effect of berries has been associated with their high content of phenolic compounds, which are particularly diverse in these fruits and include low-molecular-weight phenolic acids, such as hydroxycinnamic acids and hydroxybenzoic acids; condensed tannins; proanthocyanidins; and flavonoids such as anthocyanins (in high content) and flavonols (Leitão, Polizello, Ito, & Spadaro, 2005; Puupponen-Pimia, Nohynek, Alakomi, & Oksman-Caldentey, 2005). Polymeric tannins and, in particular, proanthocyanidins seem to be key in providing antimicrobial activity for cranberries against pathogenic bacteria (Seeram & Heber, 2007).

Several mechanisms of action are involved in the inhibition of growth of bacteria, such as destabilization of the cytoplasmic membrane, permeabilization of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, and deprivation of the substrates required for microbial growth. *In vivo* antimicrobial activity of berries may be related to the anti-adherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection by many pathogens (Puupponen-Pimia et al., 2005).

1.3.3 Hops

The hop plant belongs to the botanical family *Cannabinaceae* (Verzele & De Keukeleire, 1991), which includes the genera *Humulus* and *Cannabis*. Resin from the flowers of the hop vine (*Humulus lupulus* L.) is used in the brewing industry to impart a desirable bitter flavor to beer. The bitter compounds in hops are classified into α-bitter acids and soluble β-bitter acids, including lupulone, colupulone, and adlupulone. The α- and β-bitter acids all have a basic alicyclic structure (2,4-cyclohexadiene-l-one) but the

analogs differ in the nature of the acyl side chain (Larson et al., 1996). The β-bitter acids component mixtures (lupulones) have been reported to have greater antimicrobial activity than the iso-α-bitter acids (humulones) (Omar, 1992). The hop β-bitter acids inhibit bacteria that do not spoil beer, including *Streptococcus* spp., *E. coli*, and *L. monocytogenes*; however, they do not inhibit lactic acid bacteria that spoil beer, including species of *Lactobacillus* and *Pediococcus* (Fernandez & Simpson, 1993; Simpson, 1993). The interaction of the bitter acids with the cytoplasmic membrane is responsible for the antimicrobial activity of hops. The dissipation of the membrane pH gradient is the cause of observed inhibition of Gram-positive bacteria (Simpson, 1993).

1.3.4 Olives

Leaves and drupes from the olive tree, *Olea europaea*, are rich in biophenols such as oleuropein, verbascoside, ligstroside, tyrosol, and hydroxytyrosol, which have shown wide antioxidant and antimicrobial properties. In particular, oleuropein has exhibited antibacterial activity against a wide variety of Gram-positive and Gram-negative human pathogenic bacterial strains (Bisignano et al., 1999; Pereira et al., 2007) and several fungi (Aziz, Farag, Mousa, & Abo-Zaid, 1997), as well as antiviral activity mostly against enveloped viruses (Bisignano et al., 1999; Fredrickson, 2000; Ma et al., 2001; Micol et al., 2005).

The molecular mechanisms underlying the wide biological activity of oleuropein are not completely understood. It is believed that oleuropein, when digested by intestinal or plant β-glucosidases, yields a glutaraldehyde-like structure that exhibits protein-denaturing properties (Konno, Hirayama, Yasui, & Nakamura, 1999). These dialdehydic forms seem to exert a more potent bactericidal activity than their respective precursors (Medina, De Castro, Romero, & Brenes, 2006). It is also believed that oleuropein, a medium-polarity molecule, can get through cell membranes, reaching intracellular targets in a similar way to hydroxytyrosol (Kohyama, Nagata, Fujimoto, & Sekiya, 1997).

1.3.5 Brassica

Mustards are members of the *Cruciferaceae* or *Brassicaceae* family, and allyl isothiocyanate is the primary component of their seed. Isothiocyanates (R—N═C═S) are derivatives of glucosinolates in cells of plants of the *Cruciferae*, or mustard, family (cabbage, kohlrabi, Brussels sprouts, cauliflower, broccoli, kale, horseradish, mustard, turnip, and rutabaga). These compounds are formed from the action of the enzyme myrosinase (thioglucoside glucohydrolase) on glucosinolates when plant tissue is injured or mechanically disrupted. In addition to the allyl side group, other side groups include ethyl, methyl, benzyl, and phenyl isothiocyanate. These compounds have been reported to be potent antimicrobials (Delaquis & Mazza, 1995; Delaquis & Sholberg, 1997).

The antimicrobial spectrum of isothiocyanates is wide, inhibiting both Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative bacteria (*Cytophaga*, *E. coli* O157:H7, *Pseudomonas corrugata*, *Salmonella* Montevideo, *Salmonella* Typhimurium, and *Serratia grimesii*), yeast (*Endomyces fibuliger*), and fungi (*A. flavus*,

Aspergillus niger, *B. cinerea*, *Cladosporium cladosporioides*, *Penicillium citrinum*, *Penicillium commune*, *Penicillium expansum*, and *Penicillium roquefort*) at concentrations in the nanograms per liter range in vapor phase and in liquid media (Davidson et al., 2013). Although the effectiveness of allyl isothiocyanates varies among bacteria, Gram-positive bacteria are more resistant than Gram-negative bacteria (Obaidat & Frank, 2009; Schirmer & Langsrud, 2010). Also, the MICs of allyl isothiocyanates against common meat spoilage bacteria are much higher (1000 μg/ml) than those of other bacteria (Schirmer & Langsrud, 2010).

While the antimicrobial mechanism of isothiocyanates has not yet been fully elucidated, it is thought to be associated with sulfhydryl-containing enzymes. The dose-dependent inhibition of thioredoxin reductase, which is a sulfhydryl enzyme essential in DNA synthesis, was reported (Luciano & Holley, 2009). Although these compounds have low sensorial detection thresholds, it was suggested that they may still serve as useful food antimicrobials because of the low amounts required to inhibit targeted microorganisms (Davidson et al., 2013). Some plant volatiles, including citrus oils and vanilla, have been shown to mask the sensory effects of allyl isothiocyanates, with no effect on antimicrobial activity (Ogawa & Isshiki, 1996).

1.3.6 Alliums—garlic, onions

Onion (*Allium cepa*) and garlic (*Allium sativum*) are well-known antimicrobial systems found in plants. They have been shown to inhibit growth and production of toxins by many microorganisms including *B. cereus*, *Clostridium botulinum* type A, *E. coli*, *Lactobacillus plantarum*, *Salmonella*, *Shigella* spp., *S. aureus*, *A. flavus*, *Candida albicans*, and species of *Cryptococcus*, *Rhodotorula*, *Saccharomyces*, *Torulopsis*, and *Trichosporon* (Davidson et al., 2013).

Allicin (diallyl thiosulfinate; thio-2-propene-1-sulfanilic acid-5-allyl ester) is the major antimicrobial component of garlic and is formed by the action of the enzyme allinase on the substrate alliin [S-(2-propenyl)-L-cysteine sulfoxide]. The reaction occurs when cells are disrupted, bringing the enzyme into the presence of its substrate. A similar reaction occurs in onion except that the substrate is [S-(1-propenyl)-L-cysteine sulfoxide], and one of the major products is thiopropanal-S-oxide. The products of this reaction are responsible for the antimicrobial activity of onion and garlic and also contribute to their flavor and odor. Allicin has been demonstrated to be inhibitory toward naturally occurring microbiota in processed meats and sausages (Kim, Nahm, & Choi, 2010; Sallam, Ishloroshi, & Samejima, 2004). The mechanism of action of allicin is most likely related to the inhibition of certain thiol-containing enzymes of microorganisms by the rapid reaction of thiosulfinates with thiol groups (Ankri & Mirelman, 1999).

1.4 Types of natural antimicrobials: microbial sources

1.4.1 Natamycin

Natamycin, also termed pimaricin, is an antifungal agent that belongs to the group of polyene macrolide antimycotics and is produced by fermentation using *Streptomyces*

natalensis (Davidson & Zivanovic, 2003). Natamycin has a high affinity for ergosterol and binds irreversibly to the ergosterol in the fungal cell membrane. This perturbs the permeability of the cell membrane, which leads to rapid leakage of essential ions and small peptides and thereby causes cell lysis (Weber, Steenson, & Delves-Broughton, 2008). Natamycin was approved in 1982 in the United States for use in cheese to inhibit mold growth, with maximum concentrations of 20 mg/l in the finished product. The MIC of natamycin against almost all foodborne fungi is less than 20 ppm, whereas the solubility of natamycin in aqueous food systems is around 40 ppm. In practice, under acceptable hygienic conditions, this concentration of dissolved natamycin was demonstrated to be sufficient to prevent fungal growth without hampering the bacterial fermentation in cheese (Stark & Roller, 2003).

1.4.2 Nisin

Nisin is categorized as a class Ia bacteriocin or lantibiotic (Klaenhammer, 1993). It contains 34 amino acids and is produced by certain strains of *Lactococcus lactis* (Kupke & Gotz, 1996). Nisin was approved in the United States in 1988 for inhibiting the growth of *C. botulinum* spores and the formation of toxins in pasteurized cheese spreads and has subsequently been applied to other foods. In Europe it was added to the food additive list in the early 1980s (E234) and is the only bacteriocin that has been approved by the World Health Organization for use as a food preservative (Sobrino-Lopez & Martin-Belloso, 2008).

Nisin acts at the cytoplasmic membrane. It interacts electrostatically with membrane phospholipids to produce transient, nonselective pores. Nisin also has been shown to exhibit a high affinity for Lipid II, the universal carrier of cell wall peptidoglycan components, which accounts for its high activity in the nanomolar range when compared with some other cationic polypeptides (Wiedemann et al., 2001). The rapid efflux of ions, amino acids, and adenosine triphosphate through the pores results in collapse of the transmembrane proton motive force and cell death (Adams, 2003). Nisin is most effective against Gram-positive vegetative bacteria and spores of *Bacillus* and *Clostridium*. Activity can also be extended to Gram-negative bacteria with the addition of the chelator EDTA (Stevens, Sheldon, Klapes, & Klaenhammer, 1991).

1.4.3 Other bacteriocins

Bacteriocins are ribosomally synthesized, heat-stable, antimicrobial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains (Cotter, Hill, & Ross, 2005). Bacteriocins generally act through depolarization of the target cell membrane or through inhibition of cell wall synthesis (Abee et al., 1995). They usually range from 30 to 60 amino acids in length and have been associated with numerous species of bacteria. However, the bacteriocins of lactic acid bacteria have received much attention in terms of food safety because of their potential for GRAS status (Settanni & Corsetti, 2008). They can be readily introduced into fermented foods without prior purification or concentration (Cotter et al., 2005), and they exhibit activity against key Gram-positive pathogens such as *L. monocytogenes* and *S. aureus* (Sobrino-Lopez

& Martin-Belloso, 2008). Although they generally do not target Gram-negative bacteria, bacteriocins may be effective against these bacteria if the outer membrane is destabilized (Stevens et al., 1991).

1.4.4 Fermentates

Microbial fermentates are food-grade ingredients or foods (e.g., milk, whey, sugars) cultured with bacteria that produce organic acids (e.g., propionates) or inhibitory peptides, such as bacteriocins. They have been used commercially in the food industry to extend the shelf life of foods and inhibit foodborne pathogens. The activity of microbial fermentates is driven by the culture used. Commonly used starter cultures are *L. lactis*, *Lactobacillus* spp., *Pediococcus* spp., and *P. freudenreichii* subsp. *shermanii* (Weber et al., 2008). Microgard®, a commercially available fermented milk product containing antimicrobial metabolites, is a potential inhibitor for Gram-negative bacteria such as *Pseudomonas*, *Salmonella*, and *Yersinia*. Microgard® 100 is effective in inhibiting Gram-negative organisms, yeasts, and molds in dairy products (Al-Zoreky, Ayres, & Sandine, 1991), whereas Microgard® 300 is effective against Gram-positive organisms (except *Listeria innocua*) in dairy products (Von Staszewski & Jagus, 2008).

1.4.5 Protective cultures

Protective cultures can exert a bioprotective or inhibitory effect against other microorganisms as a result of their competition for nutrients; production of bacteriocins or other antagonistic compounds such as organic acids, hydrogen peroxide, and enzymes; or a combination of these (Melero, Vinuesa, Diez, Jaime, & Rovira, 2013). Many species of microorganism have been used as protective cultures, including not only lactic acid bacteria (e.g., lactobacilli, streptococci, enterococci, lactococci) and bifidobacteria but also *E. coli* and species of *Bacillus*, yeasts such as *Saccharomyces*, and molds such as *Aspergillus*. However, the most common protective cultures for human consumption belong to the genera of *Lactobacillus* (e.g., *Lactobacillus casei*, *Lactobacillus acidophulus*, *Lactobacillus rhamnosus*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri*) and *Bifidobacterium* (e.g., *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium breve*) (Fliss, Hammami, & Lay, 2011; Gibson, 2008). The use of protective cultures has been gaining interest, especially in the dairy, meat, and seafood industries, because they are safe for consumption (GRAS) and naturally dominate the microbiota of many foods (Castellano, Belfiore, Fadda, & Vignolo, 2008). There are several industrial starters such as SafePro® (CHR Hansen, Denmark), Bovamine Meat Culture™ (NPC, United States), and HOLDBAC™ (Danisco, Denmark) that have been developed to control *L. monocytogenes* for the meat industry (Pilet, 2011).

1.4.6 Bacteriophages

Bacteriophages (phages) are obligate parasites, and virulent phages lyse living bacterial hosts. This lytic potential has been exploited in attempts to devise a more natural antimicrobial approach to control bacteria at the various stages of food production

(Greer, 2005). Bacteriophages are suitable (1) to prevent or reduce colonization and diseases in livestock (phage therapy), (2) to decontaminate carcasses and other raw products, such as fresh fruits and vegetables, and to disinfect equipment and contact surfaces (phage biosanitation and biocontrol), and (3) to extend the shelf life of perishable manufactured foods as natural preservatives (biopreservation). Bacteriophages should also be considered in hurdle technology in combination with different preservation methods (Leverentz et al., 2003). There are some bacteriophage preparations that have received GRAS status from the US Food and Drug Administration. The antimicrobial activity of bacteriophages has been evaluated in meat, poultry, dairy, and produce against several Gram-negative and Gram-positive bacteria (Garcia, Martinez, Obeso, & Rodríguez, 2008). Because bacteriophages rely on host bacteria to replicate, it is essential that they come in contact with their bacterial hosts; contact with host bacteria is followed by bacteriophage adsorption, which combines reversible bacteriophage binding, irreversible bacteriophage binding, and bacteriophage genome transfer into the host. These processes typically occur rapidly following a collision between a bacteriophage particle and a bacteriophage-susceptible bacterium. Bacteriophage replication within the bacterial cell and release of progeny bacteriophage cause the lysis of the target microorganism (Fieseler & Loessner, 2011). Although bacteriophages could inhibit and inactivate target foodborne pathogens, the development of phage mixtures with specificity for the target organism is required for commercial application. In addition, the ability of target organisms to develop immunity to phages needs to be considered and monitored when used by commercial processors if this hurdle is to remain viable. Last, consumers may not approve of bacteriophages added to foods as a biocontrol agent (Davidson et al., 2013).

1.5 Challenges to application of natural antimicrobials to foods

Before actually applying natural antimicrobials to a commercial food product, a number of potential issues must be addressed, including its regulatory status, toxicological and allergenic effects, cost, potential form, sensory effects, influence of food components on activity, proof of effectiveness, and absence of negative interactions with natural microflora. These challenges are discussed below.

1.5.1 Regulatory status

Marketing foods with natural antimicrobials requires approval from regulatory agencies in most countries. General food safety legislation and/or maximum limits and other restrictions (e.g., acceptable daily intake and the no observed adverse effect level) of use for specific additives are established based on their toxicological information. However, it is difficult to set a specific acceptable daily intake or no observed adverse effect level for natural compounds because of the variation of components among batches. In the United States, if a direct food additive substance does not have GRAS status under the Federal Food, Drug, and Cosmetic Act, was not presumed

to be safe based on history of use by humans (marketed before October 15, 1994) under the Dietary Supplement Health and Education Act, or has no other regulatory authorization, the safety of such ingredient/compound must be established for premarket approval through the petition process (Negi, 2012). Thus, not only do naturally occurring antimicrobials, for example, clove extract, cinnamon oil, or fermented whey, from commonly consumed plant or animal products create appealing food labels for consumers, they are also less likely to be subject to the complex regulatory requirements and approval process for use in foods compared with synthetic preservatives.

1.5.2 Toxicological and allergenic effects

Toxicological and allergenic safety of food additives is a priority and one of the most important aspects to determine whether each compound is suitable for consumption. Naturally occurring and naturally derived compounds are no exception because "natural" does not necessarily mean "safe." It is essential to study the toxicological characteristics of natural antimicrobials and their biological metabolites, possible toxicity caused by interaction with food components, and allergenicity to sensitive individuals. Consumption of particular compounds, such as those contained in spices, may not cause any adverse effects at the levels present in nature, but they might be toxic once a high concentration is used to achieve antimicrobial activity or when continuously consumed for a long period of time. For example, consumption of plant extracts with a high level of tannin may result in the inhibition of trace element and vitamin B absorption, or continuous consumption of peppers (*Capsicum*) and ginger (*Zingiber officinale*) may lead to gastroesophageal reflux (Peter & Babu, 2012). Unfortunately, the process of collecting data regarding novel antimicrobial compounds and systems for their toxicity and their acceptable thresholds for human consumption is exceptionally costly and time-consuming, thus limiting the use of most novel compounds and systems.

1.5.3 Cost

In addition to effective microbial growth inhibition and inactivation activity, a successful antimicrobial needs to have a reasonable "cost in use" (David et al., 2013). Because the addition of antimicrobials increases the cost per unit of a finished product, expenditures on an antimicrobial at its required level, inactive carriers (inert ingredients such as glycerol and maltodextrin), and enablers (such as surfactants and emulsifiers) have to justify the improved microbiological stability and quality of the products. Considering the overall economics in many cases, a prolonged shelf life of at least 2−3 days could offset the cost of an antimicrobial, depending on the type of food (Davidson & Branen, 2005; David et al., 2013). Although natural antimicrobials are often more expensive than synthetic chemicals because of the production and refinement processes and their lower stability, the consumer-friendly labeling of natural compounds is highly desirable.

1.5.4 Form of antimicrobial

Determining active component(s) of naturally occurring antimicrobials and their properties is a necessary step to gain a better understanding of how each antimicrobial

works. Physicochemical properties such as polarity, volatility, net charge, pKa, and molecular configuration directly relate to the interactions of such compounds with microorganisms as well as food components (Friedman, Henika, Levin, & Mandrell, 2004; Jung, Kim, & Joo, 2013; Schofield, Mbugua, & Pell, 2001). Moreover, a consistent antimicrobial activity can be achieved by standardizing the level of active components. Although the use of highly purified antimicrobials may potentially result in greater activity, it is not necessarily desirable to highly purify natural antimicrobials. Application of isolated components defeats the objective of keeping the food label clear of chemical ingredient nomenclature. It is also more likely that a purified compound needs to be approved by regulatory agencies before use in foods. In addition, isolation of active antimicrobial component(s) would involve more processing steps, thus increasing the cost of the antimicrobial and the finished food product. Therefore the use of effective natural antimicrobials in a form as close to their sources as possible is ultimately desirable for a "clean−label" antimicrobial application to foods.

1.5.5 Sensory effects

The influence of antimicrobials on the sensory characteristics (e.g., flavor, odor, texture, and color) and the palatability of a food is a main factor for natural antimicrobial application. Flavors or odors of natural compounds could contribute to overall flavors and odors of foods, which can be desirable or undesirable. A sensorial compatibility of selected antimicrobial(s), especially ones with an intense aroma, and type of food should be carefully considered (e.g., a garlic extract is likely not a compatible antimicrobial for milk, whereas it may be good for meat products). A high concentration of many naturally occurring compounds, such as plant essential oils, are usually required to achieve significant antimicrobial activity in foods, thus causing potentially unacceptable off-odors or off-flavors (Davidson et al., 2013). The use of multiple antimicrobials could not only positively affect antimicrobial activity, it could also be beneficial for reducing sensory impact by reducing use levels below unacceptable sensory thresholds (David et al., 2013; Nguefack et al., 2012). For example, a high concentration of mustard essential oil could cause a heat sensation on the tongue. Combinations of mustard essential oil with other antimicrobials, including citrus extract, olive extract, lauric arginate, or nisin, was found to reduce the required amount of mustard oil while maintaining control of foodborne bacteria and thus potentially minimizing sensory impact (Techathuvanan, Reyes, David, & Davidson, 2014).

1.5.6 Food component interactions

Naturally occurring substances that have been shown to have high antimicrobial activity *in vitro* often have little to no effect against microbial targets at similar concentrations when incorporated into a food system. This is because of interactions with food components (Burris, Davidson, Stewart, Zivanovic, & Harte, 2012; Hyldgaard et al., 2012). Several natural antimicrobials, such as plant essential oils, are known to be amphiphilic (partially hydrophobic and partially hydrophilic). Hydrophilic

portions of antimicrobials are necessary for a compound to solubilize in the water phase where microorganisms are present, whereas lipophilic portions are required for interaction of a compound with a microbial cell membrane comprised of phospholipids (Davidson & Branen, 2005). However, that same amphiphilic character can result in antimicrobials interacting with or being solubilized by hydrophobic components of foods, such as lipids or hydrophobic portions of proteins. This makes them unavailable to react with microorganisms. Food components, including proteins (Kyung, 2011; Von Staszewski & Jagus, 2008), lipids (Cava-Roda, Taboada-Rodríguez, Valverde-Franco, & Marín-Iniesta, 2012; Gaysinsky, Taylor, Davidson, Bruce, & Weiss, 2007; Gutierrez, Barry-Ryan, & Bourke, 2008; Rattanachaikunsopon & Phurnkhachorn, 2010), carbohydrates (Al-Baarri, Hayashi, Ogawa, & Hayakawa, 2011; Devlieghere, Vermeulen, & Debevere, 2004; Gutierrez et al., 2008), and anions and cations (Al-Nabulsi & Holley, 2006; Loeffler et al., 2014) have been shown to interact with antimicrobials, resulting in a reduced biological activity. It is therefore very critical to conduct an antimicrobial efficacy test with representative parameters of a specific food system before application.

1.5.7 Activity validation methods

One of the main needs in the field of food antimicrobials is the establishment of methods to validate antimicrobial efficacy. Except for nisin and lysozyme, there are no regulatory-approved antimicrobial activity validation methods for other food antimicrobials in the United States or internationally (Davidson & Zivanovic, 2003). If a company wishes to prove that a compound has a claimed activity and/or is still effective during storage, a validation method for antimicrobial activity has to be developed (Davidson et al., 2013). Because of various available approaches (broth/agar dilution, agar diffusion, colorimetric assay/dye reduction test, and gradient plates for detection of an inhibitory end point and a turbidimetric-based method and time−kill curves for detection of dynamic microbial growth/kill kinetics) with no existing standardized assays for evaluating antimicrobial activity, evaluation methods and criteria could differ between studies (Bassolé & Juliani, 2012; Techathuvanan et al., 2014). Although models for predicting the antimicrobial activity of compounds are available (Porto, Fernandes, & Franco, 2010), validation for antimicrobial use in specific food products is crucial. The strong *in vitro* (in microbiological medium) activity of a compound does not necessarily imply high effectiveness *in situ* (in a food system). For example, a ninefold increase of thymol concentration was needed to inhibit similar levels of *E. coli* O157:H7 and *L. monocytogenes* in 2% milk compared with in apple cider (Shah, Davidson, & Zhong, 2012). To properly evaluate antimicrobial efficacy, established test protocols should be most representative of actual food products or food processing conditions (Davidson & Harrison, 2002).

1.5.8 Influence on natural microflora

Application of antimicrobials with specific activity against particular microbial types may result in selection for other microorganisms. Therefore it is important to be aware

of sources and types of normal flora and their initial levels in a food product so that the selection of antimicrobial type and dosage is appropriate.

An ideal natural antimicrobial should not contribute to the development of resistance in target microorganisms or microflora. When exposed to unfavorable environmental factors, such as extreme temperature, low pH, presence of salt and osmotic pressure, and/or intermittent or continuous sublethal concentrations of a given antimicrobial/sanitizer, microorganisms may develop either decreased susceptibility or even resistance to an antimicrobial. Adaptation of microorganisms involves stress response/cellular defense mechanisms such as alteration of a multiple antibiotic resistance operon expression, increase in an antimicrobial efflux pump, synthesis of acid shock proteins, or alteration of the microbial cell membrane (Mani-López, García, & López-Malo, 2012; Riazi & Matthews, 2011; To, Favrin, Romanova, & Griffiths, 2002). These responses may also result in increased tolerance of the microorganisms to subsequent stresses, toxin production, and/or change of microbial virulence (Davidson & Branen, 2005; Rombouts et al., 2003).

Microorganisms as part of biofilms are typically more resistant to antimicrobial treatments compared to planktonic cells. Not only do biofilms limit interaction between antimicrobial and bacterial cells, they also increase chances for microbial adaptation by allowing cell-to-cell communication, also known as "quorum sensing," to facilitate stress sensing of and response to the external environment (Fuqua & Greenberg, 2002; Parsek & Greenberg, 2005). Although there is no evidence that proper use of antimicrobials/sanitizers in food manufacturing will lead to the development of resistant microorganisms, it is possible that microbial-resistant strains may emerge (Davidson & Harrison, 2002).

1.6 Application of natural antimicrobials

Antimicrobial activity of a compound is highly dependent on its physicochemical characteristics, including polarity, hydrophilicity/hydrophobicity, volatility, and acid-dissociation property. Because of the variation in characteristics and spectra among naturally occurring antimicrobials, the difference in components and properties of foods (e.g., fat and protein content, pH, water activity, and homogeneity), process factors, and storage conditions (extrinsic factors), it is difficult to generalize about methods for antimicrobial application (Davidson & Zivanovic, 2003; Gammariello, Di Giulio, Conte, & Del Nobile, 2008). Ideally, a selected antimicrobial should be effective against the microbial target, function in the chosen food system, have no or minimal interaction with food components, and be stable during food processing. Among food systems, the least complicated product types with the minimum number of challenges for antimicrobial application are carbohydrate-based beverages, followed by bakery products, fresh produce, dairy products, and meat, poultry, and seafood products, respectively (Davidson et al., 2013).

A carbohydrate-based beverage system has the fewest challenges for microbial control using naturally occurring compounds. It is homogeneous and acidic and

comprises mainly only water and sugar with very low or no proteins or fat. Therefore, antimicrobials can be dispersed evenly throughout the product without a considerable amount of antimicrobial loss from the interaction with proteins or fats. The low pH of products often enhances antimicrobial activity (Tserennadmid et al., 2011). However, antimicrobial activity may be reduced because of the presence of simple and/or complex carbohydrates in beverages (Devlieghere et al., 2004; Gutierrez et al., 2008). Essential oils of clary sage, juniper, lemon, and marjoram were shown to have antiyeast activities against *Geotrichum candidum*, *Pichia anomala*, *S. cerevisiae*, and *Schizosaccharomyces pombe* in apple juice by extending the lag phases of microorganisms (Tserennadmid et al., 2011). The same study demonstrated that cloudy apple juice has a protective effect for yeasts when compared with clear apple juice.

Bakery products, such as cakes and breads, are usually low in proteins; however, their nonhomogeneous structure and neutral pH could limit the performance of antimicrobials. These types of products typically are only subject to fungal spoilage on the surface. Because many natural compounds, such as plant extracts, have proven activity against fungi (Kocevski et al., 2013; Masih, Peter, & Tripathi, 2014; Phillips, Laird, & Allen, 2012), the potential application of such compounds in bakery products is promising.

Fruits and vegetables are similar for their low level of fats and proteins and the presence of surface contamination. However, fruits are typically acidic, which is an advantage when applying natural antimicrobials, whereas vegetables have higher pH values. Thus the efficacy of natural antimicrobials in vegetables is often reduced, which needs to be considered. Natural antimicrobials would most likely be applied to fresh fruits and vegetables in the form of a rinse or spray. Because fresh fruits and vegetables often have rough surfaces that harbor soil and microbial contaminants, inconsistent inactivation or inhibition of microorganisms by antimicrobials often occurs (Massey, Hettiarachchy, Martin, & Ricke, 2013). Many antimicrobial-incorporated rinse/spray/wash techniques, vapor treatments, and edible coatings have been studied and developed for treatments to fruits and vegetables after harvest to improve food safety and control spoilage (González-Aguilar, Ayala-Zavala, Olivas, De La Rosa, & Álvarez-Parrilla, 2010; Massey et al., 2013; Phillips et al., 2012; Valencia-Chamorro, Palou, Del Río, & Pérez-Gago, 2011).

There are many challenges for antimicrobial use in dairy products. Their high pH and water activity provide a favorable environment for microorganisms. Dairy products contain high levels of protein, fat, and divalent cations, which can interfere with antimicrobials interacting with targets. For example, the MIC of essential oil of lemon against *G. candidum* was 0.75 μl/ml in microbiological medium, whereas the MIC in skim milk was >4 μl/ml (Tserennadmid et al., 2011). The efficacy of antimicrobials also has been shown to decrease when the fat concentration in dairy products increases (Cava-Roda et al., 2012; Gaysinsky et al., 2007). A significant decrease of antimicrobial activity of vanillin against *L. monocytogenes* and *E. coli* O157:H7 occurred in milk with higher fat content (whole milk) when compared with semiskim and skim milk (Cava-Roda et al., 2012). To improve activity, an encapsulation

technique was proposed as an antimicrobial delivery system for application in milk (Gaysinsky et al., 2007). The study showed that eugenol microemulsions (0.5% eugenol encapsulated in surfactant micelles) were effective against *L. monocytogenes* and *E. coli* O157:H7 in milk products.

Meat, poultry, and seafood products are likely the most challenging food systems for antimicrobial application because they are nonhomogeneous, high in pH, and high in protein and fat. These intrinsic factors are unfavorable for most antimicrobials, especially natural compounds, but favorable to the microorganisms. To enhance the efficacy of antimicrobials, several packaging technologies (e.g., vacuum packaging and modified atmosphere packaging) have been studied for extending the shelf life of meats (Ntzimani, Giatrakou, & Savvaidis, 2010). Dipping products in nisin and yogurt was shown to prolong the shelf life of refrigerated chicken legs to 6 days (Göğüş, Bozoglu, & Yurdugul, 2004). By using a modified atmosphere packaging with nisin and EDTA, fresh chicken was better preserved, with an extended shelf life of 14 days under refrigeration (Economou, Pournis, Ntzimani, & Savvaidis, 2009). Although microbial contamination typically occurs on the surface of meat and poultry, cutting and grinding result in physical changes of the matrices and spread contaminants throughout the products. This adversely affects the performance of natural antimicrobials by further hindering interaction with microbial targets compared with large cuts.

The use of multiple antimicrobials (especially ones with different mechanisms of actions) and/or antimicrobial(s) in combination with other control measures (such as low temperature, pressure, mild heat, low pH, and low water activity) may result in synergistic activity or create multiple hurdles that are more difficult or impossible for microorganisms to overcome (García-García, López-Malo, & Palou, 2011; Sivarooban, Hettiarachchy, & Johnson, 2008; Somolinos, García, Condón, Mackey, & Pagán, 2010). This can benefit the food industry in terms of cost and/or sensory optimization of selected antimicrobials while maintaining microbiological safety and quality. Advanced technologies for antimicrobial delivery in foods, such as encapsulation and bioactive packaging, also have been developed to enhance the antimicrobial efficacy and applicability of naturally occurring compounds (Donsi, Annunziata, Sessa, & Ferrari, 2011; Gaysinsky et al., 2007; Shah et al., 2012; Taylor, Davidson, Bruce, & Weiss, 2005; Weiss, Zhong, Harte, & Davidson, 2014).

1.7 Conclusions

There are great many unknowns and challenges to applying natural antimicrobials to food products. Effective use of these compounds requires a working knowledge of the characteristics and susceptibilities of the target microorganisms as well as interfering product components, physicochemical factors, and storage conditions. Natural antimicrobials will not likely serve as “magic bullets” to eliminate all spoilage and safety issues associated with foods. However, they can be used in an overall hurdle approach to improving both the safety and shelf life of food products on the market.

References

Abee, T., Krockel, L., & Hill, C. (1995). Bacteriocins: Modes of action and potentials in food preservation and control of food poisoning. *International Journal of Food Microbiology*, *28*, 169−185.

Adams, M. (2003). Nisin in multifactorial food preservation. In S. Roller (Ed.), *Natural antimicrobials for the minimal processing of foods*. Boca Raton, FL: CRC Press.

Al-Baarri, A. N., Hayashi, M., Ogawa, M., & Hayakawa, S. (2011). Effects of mono- and disaccharides on the antimicrobial activity of bovine lactoperoxidase system. *Journal of Food Protection*, *74*, 134−139.

Al-Nabulsi, A. A., & Holley, R. A. (2006). Enhancing the antimicrobial effects of bovine lactoferrin against *Escherichia coli* O157:H7 by cation chelation, NaCl and temperature. *Journal of Applied Microbiology*, *100*, 244−255.

Al-Zoreky, N., Ayres, J., & Sandine, W. (1991). Antimicrobial activity of Microgard™ against food spoilage and pathogenic microorganisms. *Journal of Dairy Science*, *74*, 758−763.

Alma, M. H., Ertas, M., Nitz, S., & Kollmannsberger, H. (2007). Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum L.*). *BioResources*, *2*, 265−269.

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection*, *1*, 125−129.

Arseneault, M., BéDard, S., Boulet-Audet, M., & PéZolet, M. (2010). Study of the interaction of lactoferricin B with phospholipid monolayers and bilayers. *Langmuir*, *26*, 3468−3478.

Aziz, N., Farag, S., Mousa, L., & Abo-Zaid, M. (1997). Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios*, *93*, 43−54.

Babu, R., Varshney, M., & Sogi, D. (2004). Preservation of raw milk with lactoperoxidase system. *Journal of Food Science and Technology (Mysore)*, *41*, 42−46.

Bagamboula, C., Uyttendaele, M., & Debevere, J. (2003). Antimicrobial effect of spices and herbs on *Shigella sonnei* and *Shigella flexneri*. *Journal of Food Protection*, *66*, 668−673.

Barrett, N. E., Grandison, A. S., & Lewis, M. J. (1999). Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. *Journal of Dairy Research*, *66*, 73−80.

Bassolé, I. H. N., & Juliani, H. R. (2012). Essential oils in combination and their antimicrobial properties. *Molecules*, *17*, 3989−4006.

Belaqziz, R., Bahri, F., Romane, A., Antoniotti, S., Fernandez, X., & Duñach, E. (2013). Essential oil composition and antibacterial activity of the different parts of *Thymus maroccanus Ball*: an endemic species in Morocco. *Natural Product Research*, *27*, 1700−1704.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., & Tomita, M. (1992). Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *Journal of Applied Microbiology*, *73*, 472−479.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions *Allium cepa* and garlic *Allium sativum*. *Lebensmittel Wissenschaft Und Technologie − Food Science and Technology*, *37*, 263−268.

Bisignano, G., Tomaino, A., Cascio, R. L., Crisafi, G., Uccella, N., & Saija, A. (1999). On the in vitro antimicrobial activity of oleuropein and hydroxytyrosol. *Journal of Pharmacy and Pharmacology*, *51*, 971−974.

Branen, J., & Davidson, P. (2000). Activity of hydrolysed lactoferrin against foodborne pathogenic bacteria in growth media: the effect of EDTA. *Letters in Applied Microbiology*, *30*, 233−237.

Burris, K. P., Davidson, P. M., Stewart, C. N., Zivanovic, S., & Harte, F. M. (2012). Aqueous extracts of yerba mate (*Ilex paraguariensis*) as a natural antimicrobial against *Escherichia coli* O157:H7 in a microbiological medium and pH 6.0 apple juice. *Journal of Food Protection*, *75*, 753−757.

Buzby, J. C., Wells, H. F., & Hyman, J. (2014). *The estimated amount, value, and calories of postharvest food losses at the retail and consumer levels in the United States*. Economic information bulletin. U.S. Department of Agriculture, Economic Research Service.

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*, 483−499.

Cava-Roda, R. M., Taboada-Rodríguez, A., Valverde-Franco, M. T., & Marín-Iniesta, F. (2012). Antimicrobial activity of vanillin and mixtures with cinnamon and clove essential oils in controlling *Listeria monocytogenes* and *Escherichia coli* O157: H7 in milk. *Food and Bioprocess Technology*, *5*, 2120−2131.

Chatterjee, S., Adhya, M., Guha, A., & Chatterjee, B. (2005). Chitosan from *Mucor rouxii*: production and physico-chemical characterization. *Process Biochemistry*, *40*, 395−400.

Côté, J., Caillet, S., Doyon, G., Dussault, D., Sylvain, J.-F., & Lacroix, M. (2011). Antimicrobial effect of cranberry juice and extracts. *Food Control*, *22*, 1413−1418.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*, *3*, 777−788.

Craig, W. J. (1999). Health-promoting properties of common herbs. *The American Journal of Clinical Nutrition*, *70*, 491s−499s.

David, J. R. D., Steenson, L. R., & Davidson, P. M. (2013). Expectations and applications of natural antimicrobials to foods: a guidance document for users, suppliers, research and development and regulatory agencies. *Food Protection Trends*, *33*, 238−247.

Davidson, P. M., & Branen, A. L. (2005). Antimicrobials in foods − an introduction. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in food* (3rd ed.). Boca Raton, FL: CRC Press.

Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annual Review of Food Science and Technology*, *4*, 163−190.

Davidson, P. M., & Harrison, M. A. (2002). Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology*, *56*, 69−78.

Davidson, P. M., Taylor, T. M., & Schmidt, S. E. (2012). Chemical preservatives and natural antimicrobial compounds. In M. P. Doyle, & R. L. Buchanan (Eds.), *Food microbiology: Fundamentals and frontiers* (4th ed.). Washington, DC: American Society for Microbiology.

Davidson, P. M., & Zivanovic, S. (2003). The use of natural antimicrobials. In P. Zeuthen, & L. Bøgh-Sørensen (Eds.), *Food preservation techniques*. Cambridge, UK: Woodhead Publishing Ltd.

Deans, S., Noble, R., Hiltunen, R., Wuryani, W., & Penzes, L. (1995). Antimicrobial and antioxidant properties of *Syzygium aromaticum* (L.) Merr. & Perry: impact upon bacteria, fungi and fatty acid levels in ageing mice. *Flavour and Fragrance Journal*, *10*, 323−328.

Delaquis, P. J., & Mazza, G. (1995). Antimicrobial properties of isothiocyanates in food preservation. *Food Technology*, *49*, 73−79.

Delaquis, P. J., & Sholberg, P. L. (1997). Antimicrobial activity of gaseous allyl isothiocyanate. *Journal of Food Protection*, *60*, 943−947.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703−714.

Donsi, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *Lebensmittel Wissenschaft Und Technologie − Food Science and Technology*, *44*, 1908−1914.

Dorman, H., & Deans, S. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*, 308−316.

Economou, T., Pournis, N., Ntzimani, A., & Savvaidis, I. (2009). Nisin−EDTA treatments and modified atmosphere packaging to increase fresh chicken meat shelf-life. *Food Chemistry*, *114*, 1470−1476.

EFSA Panel on Food Additives and Nutrient Sources Added to Food. (2012). Guidance for submission for food safety additives. *European Food Safety Authority Journal*, *10*, 60.

Elliot, R., Mclay, J., Kennedy, M., & Simmonds, R. (2004). Inhibition of foodborne bacteria by the lactoperoxidase system in a beef cube system. *International Journal of Food Microbiology*, *91*, 73−81.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin—a multifunctional protein with antimicrobial properties. *Molecular Immunology*, *40*, 395−405.

Fernandez, J. L., & Simpson, W. (1993). Aspects of the resistance of lactic acid bacteria to hop bitter acids. *Journal of Applied Microbiology*, *75*, 315−319.

Fieseler, L., & Loessner, M. J. (2011). Bacteriophages and food safety. In C. Lacroix (Ed.), *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation*. Cambridge, UK: Woodhead Publishing.

Fliss, I., Hammami, R., & Lay, C. L. (2011). Biological control of human digestive microbiota using antimicrobial cultures and bacteriocin. In C. Lacroix (Ed.), *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation*. Cambridge, UK: Woodhead Publishing.

Fredrickson, W. R. (2000). Method and composition for antiviral therapy. US Patent 6,117,844.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2004). Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157: H7 and *Salmonella enterica* in apple juice. *Journal of Agricultural and Food Chemistry*, *52*, 6042−6048.

Fröhlich-Wyder, M., & Bachmann, H. (2004). Cheeses with propionic acid fermentation. *Cheese: Chemistry, Physics and Microbiology*, *2*, 141−156.

Fuqua, C., & Greenberg, E. P. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nature Reviews Molecular Cell Biology*, *3*, 685−695.

Gammariello, D., Di Giulio, S., Conte, A., & Del Nobile, M. A. (2008). Effects of natural compounds on microbial safety and sensory quality of Fior di Latte cheese, a typical Italian cheese. *Journal of Dairy Science*, *91*, 4138−4146.

García-García, R., López-Malo, A., & Palou, E. (2011). Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. *Journal of Food Science*, *76*, M95−M100.

Garcia, P., Martinez, B., Obeso, J., & Rodríguez, A. (2008). Bacteriophages and their application in food safety. *Letters in Applied Microbiology*, *47*, 479−485.

Gaysinsky, S., Taylor, T. M., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157:H7. *Journal of Food Protection*, *70*, 2631−2637.

Gibis, M., & Weiss, J. (2012). Antioxidant capacity and inhibitory effect of grape seed and rosemary extract in marinades on the formation of heterocyclic amines in fried beef patties. *Food Chemistry*, *134*, 766−774.

Gibson, G. R. (2008). Prebiotics as gut microflora management tools. *Journal of Clinical Gastroenterology*, *42*, S75−S79.

Göğüş, U., Bozoglu, F., & Yurdugul, S. (2004). The effects of nisin, oil−wax coating and yogurt on the quality of refrigerated chicken meat. *Food Control*, *15*, 537−542.

González-Aguilar, G. A., Ayala-Zavala, J., Olivas, G., De La Rosa, L., & Álvarez-Parrilla, E. (2010). Preserving quality of fresh-cut products using safe technologies. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *5*, 65−72.

Greenberg, J., & Elliott, C. (2009). A cold cut crisis: Listeriosis, Maple Leaf Foods, and the politics of apology. *Canadian Journal of Communication*, *34*, 189−204.

Greer, G. G. (2005). Bacteriophage control of foodborne bacteria. *Journal of Food Protection*, *68*, 1102−1111.

Gutierrez, J., Barry-Ryan, C., & Bourke, R. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, *124*, 91−97.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*, *22*, 273−292.

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology*, *3*, 12.

Johnson, E. A., & Larson, A. E. (2005). Lysozyme. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in food* (3rd ed.). Boca Raton, FL: CRC Press.

Jones, E., Smart, A., Bloomberg, G., Burgess, L., & Millar, M. (1994). Lactoferricin, a new antimicrobial peptide. *Journal of Applied Bacteriology*, *77*, 208−214.

Jridi, M., Hajji, S., Ayed, H. B., Lassoued, I., Mbarek, A., Kammoun, M., et al. (2014). Physical, structural, antioxidant and antimicrobial properties of gelatin−chitosan composite edible films. *International Journal of Biological Macromolecules*, *67*, 373−379.

Jung, E. K., Kim, Y. J., & Joo, N. (2013). Physicochemical properties and antimicrobial activity of Roselle (*Hibiscus sabdariffa* L.). *Journal of the Science of Food and Agriculture*, *93*, 3769−3776.

Kantor, L. S., Lipton, K., Manchester, A., & Oliveira, V. (1997). Estimating and addressing America's food losses. *Food Review*, *20*, 2−12.

Kim, Y., Nahm, B., & Choi, I. (2010). An evaluation of the antioxidant and antimicrobial effectiveness of different forms of garlic and BHA in emulsion-type sausages during refrigerated storage. *Journal of Muscle Foods*, *21*, 813−825.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, *12*, 39−85.

Kocevski, D., Du, M. Y., Kan, J. Q., Jing, C. J., Lacanin, I., & Pavlovic, H. (2013). Antifungal effect of *Allium tuberosum, Cinnamomum cassia*, and *Pogostemon cablin* essential oils and their components against population of *Aspergillus* species. *Journal of Food Science*, *78*, M731−M737.

Kohyama, N., Nagata, T., Fujimoto, S.-I., & Sekiya, K. (1997). Inhibition of arachidonate lipoxygenase activities by 2-(3, 4-dihydroxyphenyl) ethanol, a phenolic compound from olives. *Bioscience, Biotechnology, and Biochemistry*, *61*, 347−350.

Konno, K., Hirayama, C., Yasui, H., & Nakamura, M. (1999). Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree. *Proceedings of the National Academy of Sciences*, *96*, 9159−9164.

Kupke, T., & Gotz, F. (1996). Expression, purification, and characterization of EpiC, an enzyme involved in the biosynthesis of the lantibiotic epidermin, and sequence analysis of *Staphylococcus epidermidis* epiC mutants. *Journal of Bacteriology*, *178*, 1335−1340.

Kussendrager, K. D., & van Hooijdonk, A. C. M. (2000). Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *British Journal of Nutrition*, *84*, S19−S25.

Kyung, K. H. (2011). Antimicrobial activity of volatile sulfur compounds in foods. *Volatile Sulfur Compounds in Food*, *1068*, 323−338.

Larson, A. E., Yu, R. R. Y., Lee, O. A., Price, S., Haas, G. J., & Johnson, E. A. (1996). Antimicrobial activity of hop extracts against *Listeria monocytogenes* in media and in food. *International Journal of Food Microbiology*, *33*, 195−207.

Lau, B. H. (2006). Suppression of LDL oxidation by garlic compounds is a possible mechanism of cardiovascular health benefit. *The Journal of Nutrition*, *136*, 765S−768S.

Leitão, D. P., Polizello, A. C. M., Ito, I. Y., & Spadaro, A. C. C. (2005). Antibacterial screening of anthocyanic and proanthocyanic fractions from cranberry juice. *Journal of Medicinal Food*, *8*, 36−40.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., et al. (2003). Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*, 4519−4526.

Loeffler, M., Mcclements, D., Mclandsborough, L., Terjung, N., Chang, Y., & Weiss, J. (2014). Electrostatic interactions of cationic lauric arginate with anionic polysaccharides affect antimicrobial activity against spoilage yeasts. *Journal of Applied Microbiology*, *117*(1), 28−39.

Luciano, F. B., & Holley, R. A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *International Journal of Food Microbiology*, *131*, 240−245.

Ma, S.-C., He, Z.-D., Deng, X.-L., But, P. P.-H., Ooi, V. E.-C., Xu, H.-X., et al. (2001). *In vitro* evaluation of secoiridoid glucosides from the fruits of *Ligustrum lucidum* as antiviral agents. *Chemical and Pharmaceutical Bulletin*, *49*, 1471−1473.

Mani-López, E., García, H., & López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, *45*, 713−721.

Masih, H., Peter, J. K., & Tripathi, P. (2014). A comparative evaluation of antifungal activity of medicinal plant extracts and chemical fungicides against four plant pathogens. *International Journal of Current Microbiology and Applied Sciences*, *3*, 97−109.

Massey, L. M., Hettiarachchy, N. S., Martin, E. M., & Ricke, S. C. (2013). Electrostatic spray of food-grade organic acids and plant extract to reduce *Escherichia coli* O157: H7 on fresh-cut cantaloupe cubes. *Journal of Food Safety*, *33*, 71−78.

Medina, E., De Castro, A., Romero, C., & Brenes, M. (2006). Comparison of the concentrations of phenolic compounds in olive oils and other plant oils: correlation with antimicrobial activity. *Journal of Agricultural and Food Chemistry*, *54*, 4954−4961.

Melero, B., Vinuesa, R., Diez, A., Jaime, I., & Rovira, J. (2013). Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere. *Poultry Science*, *92*, 1108−1116.

Micol, V., Caturla, N., Pérez-Fons, L., Más, V., Pérez, L., & Estepa, A. (2005). The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antiviral Research*, *66*, 129−136.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, *156*, 7−17.

Nguefack, J., Tamgue, O., Dongmo, J. L., Dakole, C., Leth, V., Vismer, H., et al. (2012). Synergistic action between fractions of essential oils from *Cymbopogon citratus, Ocimum gratissimum* and *Thymus vulgaris* against *Penicillium expansum*. *Food Control*, *23*, 377−383.

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science*, *264*, 382−388.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, *67*, 593−656.

Ntzimani, A. G., Giatrakou, V. I., & Savvaidis, I. N. (2010). Combined natural antimicrobial treatments (EDTA, lysozyme, rosemary and oregano oil) on semi cooked coated chicken meat stored in vacuum packages at 4° C: microbiological and sensory evaluation. *Innovative Food Science and Emerging Technologies*, *11*, 187−196.

Obaidat, M. M., & Frank, J. F. (2009). Inactivation of *Salmonella* and *Escherichia coli* O157: H7 on sliced and whole tomatoes by allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. *Journal of Food Protection*, *72*, 315−324.

Ogawa, T., & Isshiki, K. (1996). Antimicrobial activity of volatiles from edible herbs. *Journal of the Japanese Society for Food Science and Technology (Japan)*, *43*(5), 535−540.

Omar, M. (1992). Phenolic compounds in botanical extracts used in foods, flavors, cosmetics, and pharmaceuticals. In C. Ho, C. Lee, & M. Huang (Eds.), *Phenolic compounds in food and their effects on health I. Analysis, occurrence, and chemistry*. Washington, DC: ACS.

Pandey, A., Bringel, F., & Meyer, J.-M. (1994). Iron requirement and search for siderophores in lactic acid bacteria. *Applied Microbiology and Biotechnology*, *40*, 735−739.

Parry-Hanson, A., Jooste, P., & Buys, E. (2009). The influence of lactoperoxidase, heat and low pH on survival of acid-adapted and non-adapted *Escherichia coli* O157: H7 in goat milk. *International Dairy Journal*, *19*, 417−421.

Parsek, M. R., & Greenberg, E. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiology*, *13*, 27−33.

Pereira, A. P., Ferreira, I. C., Marcelino, F., Valentão, P., Andrade, P. B., Seabra, R., et al. (2007). Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. *Cv.* Cobrançosa) leaves. *Molecules*, *12*, 1153−1162.

Perumalla, A., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—Potential applications in food safety and quality. *Food Research International*, *44*, 827−839.

Peter, K. V., & Babu, K. N. (2012). Introduction to herbs and spices: medicinal uses and sustainable production. In K. V. Peter (Ed.), *Handbook of herbs and spices* (2nd ed.). Cambridge, UK: Woodhead Publishing Limited.

Phillips, C., Laird, K., & Allen, S. C. (2012). The use of Citri-V™®-an antimicrobial citrus essential oil vapour for the control of *Penicillium chrysogenum, Aspergillus niger* and *Alternaria alternata in vitro* and on food. *Food Research International*, *47*, 310−314.

Pilet, M. F. (2011). Applications of protective cultures, bacteriocins, and bacteriophages in fresh seafood, and seafood products. In C. Lacroix (Ed.), *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation*. Cambridge, UK: Woodhead Publishing.

Pino, J. A., Marbot, R., Agüero, J., & Fuentes, V. (2001). Essential oil from buds and leaves of clove (*Syzygium aromaticum* (L.) Merr. et Perry) grown in Cuba. *Journal of Essential Oil Research*, *13*, 278−279.

Porto, W., Fernandes, F., & Franco, O. (2010). An SVM model based on physicochemical properties to predict antimicrobial activity from protein sequences with cysteine knot motifs. *Advances in Bioinformatics and Computational Biology*, 59−62.

Puupponen-Pimia, R., Nohynek, L., Alakomi, H. L., & Oksman-Caldentey, K. M. (2005). Bioactive berry compounds − novel tools against human pathogens. *Applied Microbiology and Biotechnology*, *67*, 8−18.

Pyrgotou, N., Giatrakou, V., Ntzimani, A., & Savvaidis, I. N. (2010). Quality assessment of salted, modified atmosphere packaged rainbow trout under treatment with oregano essential oil. *Journal of Food Science*, *75*, M406−M411.

Rahman, M. S. (2007). Allicin and other functional active components in garlic: health benefits and bioavailability. *International Journal of Food Properties*, *10*, 245−268.

Rattanachaikunsopon, P., & Phurnkhachorn, P. (2010). Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. *Journal of Bioscience and Bioengineering*, *110*, 614−619.

Riazi, S., & Matthews, K. R. (2011). Failure of foodborne pathogens to develop resistance to sanitizers following repeated exposure to common sanitizers. *International Biodeterioration & Biodegradation*, *65*, 374−378.

Rombouts, F. M., Notermans, S. H. W., & Abee, N. T. (2003). Food preservation − future prospects. In J. Russell, & G. W. Gould (Eds.), *Food preservatives* (2nd ed.). New York, NY: Kluwer Academic/Plenum Publishers.

Sallam, K. I., Ishloroshi, M., & Samejima, K. (2004). Antioxidant and antimicrobial effects of garlic in chicken sausage. *Lebensmittel Wissenschaft Und Technologie − Food Science and Technology*, *37*, 849−855.

Sashiwa, H., & Aiba, S. I. (2004). Chemically modified chitin and chitosan as biomaterials. *Progress in Polymer Science*, *29*, 887−908.

Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V., & Hoekstra, R. M. (2011). Foodborne illness acquired in the United States—unspecified agents. *Emerging Infectious Diseases*, *17*, 16.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States − major pathogens. *Emerging Infectious Diseases*, *17*, 7−15.

Schirmer, B. C., & Langsrud, S. (2010). Evaluation of natural antimicrobials on typical meat spoilage bacteria *in vitro* and in vacuum-packed pork meat. *Journal of Food Science*, *75*, M98−M102.

Schofield, P., Mbugua, D. M., & Pell, A. N. (2001). Analysis of condensed tannins: a review. *Animal Feed Science and Technology*, *91*, 21−40.

Seeram, N. P., & Heber, D. (2007). Impact of berry phytochemicals on human health: effects beyond antioxidation. *Antioxidant Measurement and Applications*, *956*, 326−336.

Settanni, L., & Corsetti, A. (2008). Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology*, *121*, 123−138.

Shah, B., Davidson, P. M., & Zhong, Q. (2012). Nanocapsular dispersion of thymol for enhanced dispersibility and increased antimicrobial effectiveness against *Escherichia coli* O157: H7 and *Listeria monocytogenes* in model food systems. *Applied and Environmental Microbiology*, *78*, 8448−8453.

Shin, K., Yamauchi, K., Teraguchi, S., Hayasawa, H., Tomita, M., Otsuka, Y., et al. (1998). Antibacterial activity of bovine lactoferrin and its peptides against enterohaemorrhagic *Escherichia coli* O157:H7. *Letters in Applied Microbiology*, *26*, 407−411.

Simpson, W. J. (1993). Studies on the sensitivity of lactic acid bacteria to hop bitter acids. *Journal of the Institute of Brewing*, *99*, 405−411.

Sivarooban, T., Hettiarachchy, N., & Johnson, M. (2008). Physical and antimicrobial properties of grape seed extract, nisin, and EDTA incorporated soy protein edible films. *Food Research International*, *41*, 781−785.

Sobrino-Lopez, A., & Martin-Belloso, O. (2008). Enhancing the lethal effect of high-intensity pulsed electric field in milk by antimicrobial compounds as combined hurdles. *Journal of Dairy Science*, *91*, 1759−1768.

Sofos, J. N., Beuchat, L. R., Davidson, P. M., & Johnson, E. A. (1998). Naturally occurring antimicrobials in food. *Regulatory Toxicology and Pharmacology*, *28*, 71−72.

Somolinos, M., García, D., Condón, S., Mackey, B., & Pagán, R. (2010). Inactivation of *Escherichia coli* by citral. *Journal of Applied Microbiology*, *108*, 1928−1939.

Stark, J., & Roller, S. (2003). Natamycin: an effective fungicide for food and beverages. In S. Roller (Ed.), *Natural antimicrobials for the minimal processing of foods*. Cambridge, UK: Woodhead Publishing Ltd.

Stevens, K. A., Sheldon, B. W., Klapes, N. A., & Klaenhammer, T. R. (1991). Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. *Applied and Environmental Microbiology*, *57*, 3613−3615.

Stopforth, J., Skandamis, P., Davidson, P., & Sofos, J. (2005). Naturally occurring compounds − animal sources. In P. Davidson, J. Sofos, & A. Branen (Eds.), *Antimicrobials in food* (3rd ed.). New York, NY: Taylor & Francis.

Stopforth, J., Sofos, J. N., & Busta, F. F. (2005). Sorbic acid and sorbates. In P. M. Davdison, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in food* (3rd ed.). Boca Raton, FL: CRC Press.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Yamauchi, K., Takase, M., et al. (1995). *Toxoplasma gondii*: parasiticidal effects of bovine lactoferricin against parasites. *Experimental Parasitology*, *81*, 614−617.

Taylor, T. M., Davidson, P. M., Bruce, B. D., & Weiss, J. (2005). Liposomal nanocapsules in food science and agriculture. *Critical Reviews in Food Science and Nutrition*, *45*, 587−605.

Techathuvanan, C., Reyes, F., David, J. R., & Davidson, P. M. (2014). Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. *Journal of Food Protection*, *77*, 269−275.

To, M. S., Favrin, S., Romanova, N., & Griffiths, M. W. (2002). Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, *68*, 5258−5264.

Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., & Kawase, K. (1991). Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *Journal of Dairy Science*, *74*, 4137−4142.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., et al. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy*, *49*, 2474−2478.

Tserennadmid, R., Takó, M., Galgóczy, L., Papp, T., Pesti, M., Vágvölgyi, C., et al. (2011). Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *International Journal of Food Microbiology*, *144*, 480−486.

Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology*, *68*, 1561−1568.

Valencia-Chamorro, S. A., Palou, L., Del Río, M. A., & Pérez-Gago, M. B. (2011). Antimicrobial edible films and coatings for fresh and minimally processed fruits and vegetables: a review. *Critical Reviews in Food Science and Nutrition*, *51*, 872−900.

Verzele, M., & De Keukeleire, D. (1991). Chemistry and analysis of hop and beer bitter acids. *Developments in food Science, Vol. 27*. Amsterdam: Elsevier.

Vig, A. P., Rampal, G., Thind, T. S., & Arora, S. (2009). Bio-protective effects of glucosinolates − a review. *Lebensmittel Wissenschaft Und Technologie − Food Science and Technology*, *42*, 1561−1572.

Vigil, A. L. M., Palou, E., & Alzamora, S. (2005). Naturally occurring compounds-plant sources. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in food* (3rd ed.). Boca Raton, FL: CRC Press.

Von Staszewski, M., & Jagus, R. J. (2008). Natural antimicrobials: effect of Microgard™ and nisin against *Listeria innocua* in liquid cheese whey. *International Dairy Journal*, *18*, 255−259.

Weber, G., Steenson, L., & Delves-Broughton, J. (2008). Antimicrobial fermentate technology. In *Proceedings of the second international symposium on natural preservatives in food, feed, and cosmetics* (pp. 79−83). The Netherlands: Amsterdam.

Weiss, J., Zhong, Q., Harte, F., & Davidson, P. M. (2014). Micro- and nanoparticles for controlling microorganisms in foods. In G. Pabst, N. Kucerka, M.-P. Nieh, & J. Katsaras (Eds.), *Liposomes, lipid bilayers and model membranes: From basic research to technology*. Boca Raton, FL: CRC PRess.

Wiedemann, I., Breukink, E., Van Kraaij, C., Kuipers, O. P., Bierbaum, G., De Kruijff, B., et al. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry*, *276*, 1772−1779.

World Health Organization. (2002). *The world health report 2002: Reducing risks, promoting healthy life*. Geneva: WHO.

Wu, V. C. H., Qiu, X. J., Bushway, A., & Harper, L. (2008). Antibacterial effects of American cranberry (*Vaccinium macrocarpon*) concentrate on foodborne pathogens. *Lebensmittel Wissenschaft Und Technologie − Food Science and Technology*, *41*, 1834−1841.

Zivanovic, S., Chi, S., & Draughon, A. F. (2005). Antimicrobial activity of chitosan films enriched with essential oils. *Journal of Food Science*, *70*, M45−M51.

Plant extracts as antimicrobials in food products: types

2

R. Gyawali, S.A. Hayek, S.A. Ibrahim
North Carolina A&T State University, Greensboro, NC, USA

2.1 Introduction

Several food-preservation techniques have traditionally been utilized to control microorganisms in food. These preservation techniques include chilling, freezing, water activity reduction, nutrient restriction, acidification, modified atmosphere packaging (MAP), fermentation, and nonthermal physical treatments or the addition of synthetic antimicrobials (Davidson, 2001). Preservation methods that utilize synthetic preservatives have had wide applications in the food industry. However, in recent years, increasing concerns over negative health issues associated with the use of synthetic preservatives were noticed (Sofos & Geornaras, 2010). These increased concerns have motivated the scientific community to investigate natural antimicrobial products as an alternative. Plants are rich in natural substances with antimicrobial properties, and act as antioxidants and flavor- and color-enhancing agents that can increase organoleptic acceptability, extend the shelf life of foods, and prevent the growth of foodborne pathogens (Razavi Rohani, Moradi, Mehdizadeh, Saei-Dehkordi, & Griffiths, 2011). Additionally, most plant-derived extracts are generally recognized as safe (GRAS) (21 Code of Federal Regulations (CFR) §182, 184) (Burt, 2004).

Antimicrobial extracts obtained from plant products have thus been identified as promising alternatives to existing synthetic preservatives (Wallace, 2004). Plants are rich in a variety of antimicrobial compounds such as saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes, lactones, terpenoids, and phorbol esters (Lewis & Ausubel, 2006; Tajkarimi, Ibrahim, & Cliver, 2010). Numerous studies have been published on the antimicrobial properties of the secondary metabolites of plant products (Bajpai, Rahman, & Kang, 2008; Tajkarimi et al., 2010; Tiwari et al., 2009). Polyphenols, which are particularly flavonoid-rich plant extracts, have antibacterial activity against a range of pathogenic microorganisms (Cushnie & Lamb, 2005). Polyphenolic compounds are present in diverse structural composition, and these differences in the chemical structures of polyphenols also contribute to the antimicrobial activity (Daglia, 2012). Essential oils (EOs) from spices and herbs are GRAS and have been used as food preservatives since ancient times (Shan, Cai, Brooks, & Corke, 2007). However, in the last few decades there has been an increased interest in discovering the exact antimicrobial effect of these EOs. Several recent reviews have focused on recent reports of the antimicrobial effect of spices and herbs or EOs against foodborne pathogenic bacteria (Burt, 2004; Davidson, 2001; Hayek, Gyawali, & Ibrahim, 2013; Tajkarimi et al., 2010; Tiwari et al., 2009).

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In this chapter, we have also highlighted the potential enhancement of plant extract antimicrobial activity in the presence of minerals. Minerals such as Cu^{2+}, Zn^{2+}, and Fe^{3+} are widely found in plant products, and their antimicrobial property has been established (Gyawali and Ibrahim, 2012; Özcan, 2004). Using extracts from plant sources in combination with external mineral sources could provide stronger antimicrobial effects and a safer approach to the control of foodborne pathogens. This chapter summarizes the major plant extracts that have been investigated as food antimicrobials for their potential application in the food system.

2.2 Herbs, spices, and plant extracts as antimicrobials

Herbs, spices, and plant extracts are GRAS products that been used for centuries in the food systems as means of flavor and preservatives (Shan et al., 2007; Tiwari et al., 2009). Spices such as clove, cinnamon, oregano, and rosemary are some of the most common spices and herbs with strong antimicrobial activity. EOs obtained from these spices contain several active compounds with antimicrobial activity such as carvacrol (5-isopropyl-2-methylphenol), cinnamaldehyde (3-phenylprop-2-enal), eugenol (4-allyl-2-methoxyphenol), and camphor (Weerakkody, Caffin, Turner, & Dykes, 2010). Spices are rich in polyphenolic compounds, flavonoids, terpenoids, and other volatile compounds as antimicrobial components (Gulluce et al., 2007). Table 2.1 lists antimicrobial activity of selected plant products against foodborne pathogens and spoilage microorganisms.

Zaika (1988) grouped different spices based on the antimicrobial activity as strong (cinnamon, clove, mustard); medium (allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage, thyme); or weak (black pepper, red pepper, ginger). Cinnamon, clove, and mustard have antimicrobial activity against mycotoxigenic *Aspergillus* spp., *Aspergillus parasiticus*, *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli*, mycotoxigenic *Aspergillus*, *Shigella sonnei*, and *Shigella flexneri* (Lai & Roy, 2004). Cumin, oregano, rosemary, sage, and thyme have inhibitory properties against *S. enterica*, *L. monocytogenes*, *E. coli*, *Staphylococcus aureus*, and *Bacillus cereus*. Garlic extract has antibacterial activity against all serogroups of *E. coli*, whereas enterohemorrhagic *E. coli* (serogroup O157) and enterotoxigenic *E. coli* (serogroup O8) are more sensitive to garlic extract (Indu, Hatha, Abirosh, Harsha, & Vivekanandan, 2006). Garlic has also shown inhibitory effects against a wide range of microorganisms including *Salmonella* Typhimurium, *S. aureus*, *B. cereus*, *Bacillus subtilis*, mycotoxigenic *Aspergillus*, *Candida albicans*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* (Lai & Roy, 2004).

Gulluce et al. (2007) reported the antimicrobial properties of several species of the genus *Mentha* including peppermint (*Mentha piperita* L.) and spearmint (*Mentha spicata*) against bacteria (*Bacillus* spp., *E. coli*-A1, *Salmonella* spp., *Staphylococcus* spp.), yeast (*C. albicans*-A117), and fungi (*Aspergillus* spp., *Fusarium* spp.). Similarly, antimicrobial properties of mint against pathogenic microorganisms have also been demonstrated (Tassou, Koutsoumanis, & Nychas, 2000). Nguyen and Mittal (2007) reported 4.77 and 8.34 log CFU/ml reductions in the native flora of pasteurized tomato juice with ground mint extract at 0.1% and 1.2% (w/v), respectively, when

Table 2.1 Antimicrobial activity of selected plant products

Categories	Products	Effective against	References
Herbs and spices	Cinnamon, clove, mustard, allspice, coriander, cumin, oregano, rosemary, sage, ginger, turmeric	*Salmonella*, *E. coli*, *S. aureus*, *Yersirnia enterocolitica*, *Bacillus* spp., *Pseudomonas*, *Shiggella*, *Clostridium botulinum*, *Listeria monocytogenes*	Ceylan and Fung (2004), Zaika and Kissinger (1984)
Seeds	Grape seeds, muscadine seeds, cardamom, poppy seeds, anise seed, celery seed	*L. monocytogenes*, *S.* Typhimurium, *E. coli* O157:H7, *Campylobacter jejuni*	Ceylan and Fung (2004), Perumalla and Hettiarachchy (2011), Kim, Weng, et al. (2008)
Leaves	Cilantro, bay leaf, sweet basil, thyme, mint, lemon grass, sorrel, ruta	*L. monocytogenes*, *B. cereus*, *Salmonella enterica*, *S. aureus*	Delaquis, Stanich, Girard, and Mazza (2002), Alzoreky and Nakahara (2003), Moore-Neibel, Gerber, Patel, Friedman, and Ravishankar (2012), Shan, Cai, Brooks, and Corke (2007)
Fruits	Pomegranate, xoconostle pears, cloudberry, raspberry, strawberry	*E. coli* O157:H7, *Salmonella*	Hayek and Ibrahim (2012), Ibrahim, Bor, Song, and Tajkarimi (2011), Puupponen-Pimiä et al. (2001)
Vegetables	Bell pepper, chives, broccoli, kale	*E. coli* O157:H7, *S.* Typhimurium, *Pseudomonas aeruginosa*, *S. aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *L. monocytogenes*	Mau, Chen, and Hseih (2002), Careaga et al. (2003), Ayaz et al. (2008), Corrêa, Martin, Alencar, and Porto (2014)

Continued

Table 2.1 Continued

Categories	Products	Effective against	References
Flowers, bud	Hibiscus, rose, clove	*B. cereu*, *B. subtilis*, *E. coli*, *L. monocytogenes*, *S. aureus*, *Salmonella*, *P. aeruginosa*	Shan et al. (2007), Ulusoy, Boşgelmez-Tınaz, and Seçilmiş-Canbay (2009), Ruban and Gajalakshmi (2012)
Bark, peel	Cassia, lemon peel, pomegranate rind	*E. coli*, *Salmonella infantis P. aeruginosa*, *S.* Typhimurium, *S. aureus*	Alzoreky and Nakahara (2003); Dhanavade, Jalkute, Ghosh, and Sonawane (2011), Gould, Fielder, Kelly, and Naughton (2009)

heated at 50 °C. Only 0.74 log CFU/ml reduction of native microorganisms was achieved when tomato juice without mint was heated at 50 °C. Thongson, Davidson, Mahakarnchanakul, and Vibulsresth (2005) reported the antimicrobial effect of the Thai spice fingerroot (*Boesenbergia pandurata*) against *L. monocytogenes* in culture media by using agar dilution assay. Ethanolic fingerroot extracts at 5−10% (v/v) inhibited most of the *L. monocytogenes* strains in 24 h. A 5% fingerroot ethanol extract reduced the population of *L. monocytogenes* by 2.0 log CFU/ml in 24 h, while 10% extract caused total reduction in 9 h of incubation. Sagdic, Kuscu, Ozcan, and Ozcelik (2002) reported the antimicrobial activity of various Turkish spice extracts (cumin, laurel, myrtle, oregano, sage, and thyme) against the growth of *E. coli* O157:H7. Among these Turkish spices, only thyme showed effective results by inhibiting the bacterial growth both in paper disc assay and agitated liquid culture assay.

Garlic extract (Singh, Bernadette Falahee, & Adams, 2001) and garlic shoot juice alone or in combination with other natural preservatives (Kim, Choi, Bajpai, & Kang, 2008) have also shown antilisterial activity. Spices including basil, bay leaves, cinnamon, clove, cumin, dill, fennel, garlic, lemongrass, mint, onion, oregano, rosemary, sage, and thyme can inhibit a wide range of pathogenic bacterial species (Ceylan and Fung, 2004). Chinese chives, cinnamon, and corni fructus are used as flavoring agents, and they also have strong antimicrobial potential (Hsieh, Mau, & Huang, 2001; Mau, Chen, & Hseih, 2001). The combined extract of corni fructus, cinnamon, and Chinese chive (8:1:1, v/v/v) showed excellent antimicrobial activity against common foodborne microorganisms including bacteria (*B. subtilis*, *E. coli*, *L. monocytogenes*, *S.* Typhimurium, *S. aureus*), yeasts (*Aspergillus flavus*, *Aspergillus niger*), and molds (*Pichia membranaefaciens*, *Kloeckera apiculata*, *Debaryomyces*

hansenii) (Hsieh et al., 2001). Methanol extract of *Orthosiphon stamineus* (cat whisker) has strong inhibitory activity against *Vibrio parahaemolyticus* (Ho, Noryati, Sulaiman, & Rosma, 2010). The efficacy of this plant product was similar to the inhibitory effect produced by 5% lactic acid. Authors noted that the presence of high concentrations of rosmarinic acid in the *O. stamineus* extracts contributed to the strong antimicrobial effect.

Cinnamon powder contains cinnamaldehyde and eugenol as the major compounds with antimicrobial effects. Cinnamon was early reported for a strong antimicrobial activity against microorganisms (Zaika, 1988). Apple juice in the presence of 0.1%, 0.2%, and 0.3% (w/v) of ground cinnamon exhibited a 4.0−6.0 log CFU/ml reduction in *L. monocytogenes* after 1 h of incubation at 5 and 20 °C (Yuste & Fung, 2002). Ceylan, Fung, and Sabah (2004) demonstrated that the addition of 0.3% (w/v) cinnamon powder to pasteurized apple juice decreased the population of *E. coli* O157:H7 by 1.6 and 2.0 log CFU/ml stored at 8 and 25 °C, respectively. In the presence of 2% (w/v) cinnamon powder, the *E. coli* O157:H7 population was reduced by 2.0 log CFU/ml in unpasteurized apple cider at 42 °C (Iu, Mittal, & Griffiths, 2001).

Fermented tea has inhibitory effects against several foodborne pathogens and spoilage bacteria (Mo, Zhu, & Chen, 2008; Sreeramulu, Zhu, & Knol, 2000; Xu, Yan, & Zhu, 2005). Tea polyphenols can inhibit *Bacillus stearothermophilus*, which is a thermophilic spore-forming bacterium. The growth and development of spore-forming bacteria *B. stearothermophilus* and *Clostridium thermoaceticum* is reduced by the addition of tea polyphenols at high temperature. The strong antimicrobial activity of tea polyphenol is mainly due to the presence of epigallocatechin gallate (EGCG) (Sakanaka, Juneja, & Taniguchi, 2000). This indicates that antimicrobial compounds produced during the fermentation of tea are thermo-stable and could be used to control thermophilic spore-forming bacteria. Green tea and grape seed extracts (polyphenolic and proanthocyanidin rich compounds, respectively) have potential antimicrobial activity against major foodborne pathogens including *L. monocytogenes*, *S.* Typhimurium, *E. coli* O157:H7, and *Campylobacter jejuni* (Perumalla & Hettiarachchy, 2011). The authors have also demonstrated the synergistic antimicrobial activity of green tea and grape seed extracts when used in combination with organic acids (malic, tartaric acid, benzoic acid, etc.). Caffeine (1, 3, 7-trimethyl xanthine) is one of three methylated xanthine alkaloid derivatives present in many plant species (Ibrahim, Salameh, Phetsomphou, Yang, & Seo, 2006) and has antimicrobial activity against *E. coli* O157:H7, *Salmonella*, *Pseudomonas* spp., *S. aureus*, and several strains of the Enterobacteriaceae family (Almeida, Farah, Silva, Nunan, & Glória, 2006; Daglia, Cuzzoni, & Dacarro, 1994; Dash & Gummadi, 2008; Esimone, Okoye, Nworu, & Agubata, 2008; Gyawali, Adkins, Minor, & Ibrahim, 2014; Ibrahim et al., 2006; Ramanavičienė, Mostovojus, Bachmatova, & Ramanavičius, 2003).

The antibacterial effects of spice extracts varied in proportion to concentrations. Hara-Kudo, Kobayashi, Sugita-Konishi, and Kondo (2004) reported that amounts of phenolic compounds in spices and herbs play a major role in the antimicrobial effects. For example, garlic and clove (0.25−1%) in culture medium showed bacteriostatic and bacteriocidal towards *E. coli* O157 and *Salmonella* Enteritidis

(Leuschner & Zamparini, 2002). Cranberry powder at 1%, 2%, and 3% resulted in 2−4 log CFU/g less growth of *L. monocytogenes* compared to nitrite at 156 mg/kg (control), a commonly used ingredient in cured meats. Authors have also reported the potential use of cherry powder, lemon powder, lime powder, and grape seed extract to control *L. monocytogenes* when combined with cranberry powder (Xi, Sullivan, Jackson, Zhou, & Sebranek, 2011). Cranberry juice and cranberry concentrates have also shown antibacterial effect against *S. aureus* (Magrarinos et al., 2008; Qiu & Wu, 2007). Trans-cinnamaldehyde (0.5%) reduced the population of *Cronobacter sakazakii* to undetectable levels within 4 h of incubation at 37 °C in reconstituted infant formula (Amalaradjou, Hoagland, & Venkitanarayanan, 2009). Water-soluble muscadine seed extracts reduced the population of *C. sakazakii* (6.0 log CFU/ml) to an undetectable level within 1 h of incubation at 37 °C (Kim et al., 2009). In addition, a 5.0 log reduction of *L. monocytogenes* within 1 h by grape seed extract has been previously reported by Rhodes, Mitchell, Wilson, and Melton (2006). Grape pomace extract of 2.5%, 5%, 10%, and 20% (w/v) inhibited spoilage and pathogenic bacteria including *Aeromonas hydrophila*, *B. cereus*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *E. coli*, *E. coli* O157:H7, *Mycobacterium smegmatis*, *Proteus vulgaris*, *P. aeruginosa*, *Pseudomonas* fluorescens, *S.* Enteritidis, *S.* Typhimurium, and *S. aureus* (Özkan Sagdiç, Göktürk Baydar, & Kurumahmutoglu, 2004).

Su, Howell, and D'Souza (2012) evaluated the antimicrobial activity of grape seed extract, pomegranate polyphenols, and cranberry proanthocyanidins against two strains of methicillin-resistant *S. aureus* (MRSA). Among the three tested extracts, grape seed extract at 1 and 5 mg/ml reduced the population of MRSA by 3.0−4.0 log CFU/ml within 2 h of incubation at 37 °C. Similarly, Al-Habib, Al-Saleh, Safer, and Afzal (2010) reported the complete inhibition of MRSA at 3 mg/ml of grape seed extract when tested in laboratory media. Grape skin extract is effective against Gram-positive bacteria (*S. aureus*, *B. cereus*) and Gram-negative bacteria (*E. coli* O157:H7, *Salmonella* Infantis, *Campylobacter coli*) in culture medium (Katalinić et al., 2010). Minimum inhibitory concentrations (MICs) were observed at 0.014−0.59 mg of gallic acid where total phenols were expressed as gallic acid equivalents in grape skin extract. The presence of phenolic compounds and their derivatives, natural organic acids (tartaric and malic acids), and phenolic acids (tannic and gallic acids) may have contributed to this strong antimicrobial activity (Kim, Weng, Silva, Jung, & Marshall, 2010). The population of *Campylobacter* spp. was reduced by 5.08−6.97 log CFU/ml with 500 mg/l grape seed extract. Minimal inhibitory concentration (MIC) of 20 mg/l and a minimal bactericidal concentration (MBC) of 60 mg/l against *C. jejuni* were reported by Silván et al. (2013). Compounds in grape seed extracts such as phenolic acids, catechins, and proanthocyanidins are attributed to antimicrobial properties against *C. jejuni* (Silván et al., 2013).

Plant extracts can be used to decontaminate produce surfaces inoculated with foodborne pathogens. Several authors have proposed washing solutions for fresh produce with natural products such as grape seed extract, olive extracts, and essentials oils as alternatives to chlorine-based washing method (Bisha, Weinsetel, Brehm-Stecher, & Mendonca, 2010; Lu & Wu, 2010; Moore, Patel, Jaroni, Friedman, & Ravishankar,

2011). Orue, García, Feng, and Heredia (2013) reported that extracts from oregano leaves and from the peel and pulp of limes are as effective as chlorine or citrol in reducing *Salmonella*, *E. coli* O157:H7, and *S. sonnei* by >2.0 log cycles on leafy greens such as cilantro, parsley, and spinach. Clove extract *(Syzygium aromaticum)* can reduce the population of *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* attached to the surface of lettuce (Kim et al., 2011). Early studies have also demonstrated the effectiveness of washing fresh produce by EOs. Wan, Wilcock, and Coventry (1998) used basil EO to wash fresh lettuce against natural microbial flora, and the effectiveness was comparable to the treatment with 125 ppm chlorine. Grape seed extract (0.125%) can reduce viable cell counts for *L. monocytogenes* by approximately 2.0 log units within 2 min on tomato surfaces (Bisha et al., 2010). In addition, organic acids are GRAS chemicals that are naturally found in plants. A significant reduction in the population of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was reported from the surface of apples and lettuce when treated with organic acids (propionic acid, acetic acid, lactic acid, malic acid, and citric acid) at 1% and 2%, v/v (Park et al., 2011). Since several plant species contain various antimicrobial components including organic acids, extracts from plants have high potential applications to decontaminate fresh produce and replace chemical sanitizers. These natural antimicrobial wash solutions might offer a value-added approach to control foodborne pathogens on produce or in produce production environments.

2.3 Essential oils

EOs are aromatic oily liquids obtained from plant materials, mainly herbs and spices (Burt, 2004). EOs have antimicrobial activity against a wide range of pathogenic and spoilage microorganisms (Dadalioglu & Evrendilek, 2004). EOs have a relatively high vapor pressure and they are capable to reach the microorganisms through both liquid and gas phases. Most EO antimicrobial work has been conducted *in vitro*, using laboratory culture media (Campo & Amiot, 2000; Delaquis et al., 2002; Elgayyar, Draughon, Golden, & Mount, 2001; Griffin, Markham, & Leach, 2000; Munoz, Guevara, Palop, Tabera, & Fernández, 2009). EOs from thyme, clove, and pimenta can reduce the population of *L. monocytogenes* in peptone solution (1 g/l) at low concentration level, 0.5 ml/l (Singh, Singh, Bhunia, & Singh, 2003). The results showed that thyme and clove EOs reduced *L. monocytogenes* population from 7.17 log to 1.48 log CFU/g within 5 min of treatment time. Pimento EO caused only slight reduction in *L. monocytogenes* population, from 7.05 to 6.75 log CFU/g, at the same conditions. Similarly, *L. monocytogenes* population was reduced below the level of detection within 4 h of exposure in culture medium at 30 °C in the presence of EOs from oregano, rosemary, and laurel (Muñoz et al., 2009). The combinations of EOs from oregano with marjoram, and thyme with sage, had promising efficacy against *E. coli* and *L. monocytogenes,* respectively. However, EOs from oregano and thyme are more effective when applied individually compared to combination (Gutierrez, Barry-Ryan, & Bourke, 2008). The addition of thyme EO (0.1%) to modified atmospheric packaged chopped lamb meat resulted in significant increase in the shelf life (Karabagias, Badeka, & Kontominas, 2011). EOs from citrus fruits can inhibit the

growth of both Gram-positive and Gram-negative bacteria. The major chemical components of citrus oils is limonene which is present in many fruits in the range of 68–98% in sweet orange, 45–76% in lemon, and 32–45% in bergamot (Svoboda & Greenaway, 2003). Fisher and Phillips (2008) have reported that the direct oil and vapor from citrus oils with 85–99% volatile and 1–15% nonvolatile components could have contributed to the antimicrobial properties of these oils.

It was suggested that the presence of phenolic compounds containing polar functional groups is chiefly responsible for the antimicrobial properties of EOs (Burt, 2004). Phenolic compounds of EOs such as citrus oils extracted from lemon, olive oil (oleuropein), and tea-tree oil (terpenoids), orange, and bergamot have broader antimicrobial effects. However, there are increasing reports of nonphenolic oil compounds, which are effective against both Gram-positive and Gram-negative groups, from oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, muscadine seeds, and sage (Hayek et al., 2013). Cueva et al. (2010) grouped the antimicrobial properties of phenolic compounds based on their chemical structure. The antimicrobial effects of the phenolic acids were structure-dependent, such as benzene ring substitutions and saturated side-chain lengths greatly influencing the antimicrobial activity of the compound (Cueva et al., 2010). The components with phenolic structures, such as carvacrol, eugenol, and thymol, have higher antimicrobial activity against microorganisms. In these compounds the presence of hydroxyl groups, their number, and their position in the phenolic structure have been shown to influence the antimicrobial activity (Dorman & Deans, 2000). Some nonphenolic constituents of EOs such as allyl isothiocyanate and garlic oil are quite effective against Gram-negative bacteria (Hayek et al., 2013).

2.4 Plant extracts in combination with minerals

Plants are rich in active compounds including phytochemicals, phenols, polyphenols, essentials oils, and minerals. The antimicrobial properties of these active compounds have been reported. Minerals have been included in foods mainly as nutrients, but they could serve other biological function including antimicrobial activity. Minerals are naturally found in food and have been approved as food-enriching nutrients. However, minerals are allowed to be added only in trace amounts, which sometimes are not enough to control microbial growth. In addition, plant extracts, if used in high concentration, could affect the sensory properties of food. Combination of plant extracts and minerals could result in enhanced levels of antimicrobial activity. However, only a limited number of studies have investigated whether the antimicrobial properties of plant extracts can be enhanced with the addition of minerals. This section highlights the potential effects of plant extracts in the presence of minerals on the growth of pathogenic microorganisms. Table 2.2 lists antimicrobial activity of selected plant extracts in the presence of minerals.

Zinc sulfate ($ZnSO_4$) and iron (II) sulfate ($FeSO_4$) have strong inhibitory effect on the growth of *E. coli* (Yadav, Gite, Nilegaonkar, & Agte, 2011), indicating the inclusion of these minerals with plant extracts could further potentiate the antimicrobial

Table 2.2 Antimicrobial activity of plant extracts in combination with minerals

Plant extracts	Minerals	Antimicrobial action	References
Pomegranate rind extract	$CuSO_4$	Methicillin-resistant *Staphylococcus aureus* (MRSA)	Gould et al. (2009)
Pomegranate rind extract	$FeSO_4$	*Pseudomonas* phage	Stewart et al. (1998)
Vanilin extract	Cupric salts	*S. aureus*, *Escherichia coli*, *K. pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans*	Sivasankaran-Nair and Selwin-Joseyphus (2008)
Green tea (catechin)	Cu^{2+}	*E. coli*, *S. aureus*	Hoshino et al. (2000)
Corni fructus + cinnamon + Chinese chive	Na^{+}, Ca^{2+}, Zn^{2+}, Mg^{2+}	*Bacillus subtilis*, *E. coli*, *Listeria monocytogenes*, *Salmonella Typhimurium*	Hsieh et al. (2011)

activity. Gould et al. (2009) examined the efficacy of pomegranate rind extract in the presence of cupric sulfate against MRSA. Pomegranate rind extract in combination with copper caused a 4.0 log reduction of MRSA isolates (Gould et al., 2009). Sivasankaran-Nair and Selwin-Joseyphus (2008) demonstrated the enhancement of natural products (vanillin extract) by the addition of metal ions, especially cupric salts. The authors reported copper and vanillin extract to be the most effective treatment when tested by agar diffusion assay against microorganisms such as *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. vulgaris*, *P. aeruginosa*, and *C. albicans*. Mau et al. (2002) demonstrated the enhanced inhibitory effect of vegetable juices in the presence of metal ions (Zn^{2+}, Na^{+}, Mg^{3+}, Fe^{3+}) at 1 or 10 mM against foodborne bacteria, yeasts, and molds. Khalid, Fawad, and Ahmed (2011) reported the antimicrobial activity of black mulberry (*Morus nigra* L.) juice against *Bacillus spizizenii* and *P. aeruginosa*. The inhibitory effect was attributed to the presence of antioxidant components including trace minerals such as zinc and iron in higher amounts. Study of the antimicrobial effects of mulberry juice, which is naturally rich in K, Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}, in combination with minerals could be another approach to enhance the antimicrobial efficacy. The antimicrobial effect of metal ions (Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, or Fe^{3+}) in the presence of combined extracts of corni fructus + cinnamon + Chinese chive (8:1:1, v/v/v) was also reported (Hsieh et al.,

2001). These results support the idea of utilizing plant extracts with minerals as antimicrobial agents.

The antimicrobial activity of plant phenolics can be enhanced in combination with metal ions or vitamin C (Hoshino, Kimura, Hayakawa, Yamaji, & Ando, 2000; Iwasaki et al., 2011). The antimicrobial efficacy of catechin-copper (II) mixture in the presence of vitamin C against *S. aureus* and *E. coli* was also determined (Holloway et al., 2012). When copper (II) ions combined with plant components, bactericidal reactive oxygen species including H_2O_2 could be generated, and the antibacterial activity of plant components could be preserved by vitamin C in aqueous solutions, thereby enhancing antimicrobial efficacy (Holloway et al., 2012). In addition, the antimicrobial effect of minerals can also be enhanced in combination with organic acids. Acidic environments enhance the antibacterial activity of products including plant extracts (Boziaris, Proestos, Kapsokefalou, & Komaitis, 2011). Recently, we demonstrated the effect of Cu^{2+} ions with organic acid to decontaminate the surface of lettuce and tomatoes inoculated with *E. coli* O157:H7 (Gyawali, Ibrahim, Abu Hasfa, Smqadri, & Haik, 2011).

In a recent review, Gyawali and Ibrahim, 2012 reported an interesting approach on plant species containing several minerals and their antimicrobial action. The authors reported that minerals in plants such as Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, and Cu^{2+} could have a direct or indirect impact on the survival and growth of pathogens. These minerals are considered for the growth of lactic acid bacteria, organic acid production during fermentation, and the production of functional metabolites. The growth of lactic acid bacteria and the production of organic acids and bacteriocins could result in inhibiting the growth of the pathogenic bacteria (Gyawali and Ibrahim, 2012).

2.5 Conclusion

Natural products are of GRAS status, so any safety concerns related to their use in preventing the growth of foodborne pathogens have been minimal. However, the optimal range of plant antimicrobials that can be used in food products should be clearly established before the antimicrobials are used in complex food systems. The use of such natural plant extracts when considered in addition to other factors, such as low storage temperature, low pH, anaerobic temperatures, and organic acids, could help achieve synergistic antimicrobial effects. In addition, the efficacy of plant extracts could also be enhanced by the addition of minerals. Further work is necessary to establish the mode of antimicrobial action along with its enhancement mechanism in the presence of minerals and plant extracts.

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References

Al-Habib, A., Al-Saleh, E., Safer, A.-M., & Afzal, M. (2010). Bactericidal effect of grape seed extract on methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Toxicological Sciences*, *35*(3), 357−364.

Almeida, A. A. P., Farah, A., Silva, D. A. M., Nunan, E. A., & Glória, M. B. A. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry*, *54*(23), 8738−8743.

Alzoreky, N. S., & Nakahara, K. (2003). Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *International Journal of Food Microbiology*, *80*(3), 223−230.

Amalaradjou, M. A. R., Hoagland, T. A., & Venkitanarayanan, K. (2009). Inactivation of *Enterobacter sakazakii* in reconstituted infant formula by trans-cinnamaldehyde. *International Journal of Food Microbiology*, *129*(2), 146−149.

Ayaz, F. A., Hayirlioglu-Ayaz, S., Alpay-Karaoglu, S., Grúz, J., Valentová, K., Ulrichová, J., & Strnad, M. (2008). Phenolic acid contents of kale (*Brassica oleraceae* L. var. *acephala* DC.) extracts and their antioxidant and antibacterial activities. *Food Chemistry*, *107*(1), 19−25.

Bajpai, V. K., Rahman, A., & Kang, S. C. (2008). Chemical composition and inhibitory parameters of essential oil and extracts of *Nandina domestica* Thunb. to control food-borne pathogenic and spoilage bacteria. *International Journal of Food Microbiology*, *125*(2), 117−122.

Bisha, B., Weinsetel, N., Brehm-Stecher, B. F., & Mendonca, A. (2010). Antilisterial effects of gravinol-s grape seed extract at low levels in aqueous media and its potential application as a produce wash. *Journal of Food Protection*, *73*(2), 266−273.

Boziaris, I. S., Proestos, C., Kapsokefalou, M., & Komaitis, M. (2011). Antimicrobial effect of *Filipendula ulmaria* plant extract against selected foodborne pathogenic and spoilage bacteria in laboratory media, fish flesh and fish roe product. *Food Technology and Biotechnology*, *49*(2), 263−270.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223−253.

Campo, J. D., & Amiot, M.-J. (2000). Antimicrobial effect of rosemary extracts. *Journal of Food Protection*, *63*(10), 1359−1368.

Careaga, M., Fernández, E., Dorantes, L., Mota, L., Jaramillo, M. E., & Hernandez-Sanchez, H. (2003). Antibacterial activity of Capsicum extract against Salmonella typhimurium and *Pseudomonas aeruginosa* inoculated in raw beef meat. *International Journal of Food Microbiology*, *83*(3), 331−335.

Ceylan, E., & Fung, D. Y. (2004). Antimicrobial activity of spices. *Journal of Rapid Methods and Automation in Microbiology*, *12*(1), 1−55.

Ceylan, E., Fung, D. Y. C., & Sabah, J. R. (2004). Antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *Escherichia coli* O157:H7 in apple juice. *Journal of Food Science*, *69*(4), FMS102−FMS106.

Corrêa, C. B., Martin, J. G. P., Alencar, S. M., & Porto, E. (2014). Antilisterial activity of broccoli stems (Brassica oleracea) by flow cytometry. *International Food Research Journal*, *21*(1), 395−399.

Cueva, C., Moreno-Arribas, M., Martín-Álvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., et al. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology*, *161*(5), 372−382.

Cushnie, T. P., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *26*(5), 343−356.

Dadalioglu, I., & Evrendilek, G. A. (2004). Chemical compositions and antibacterial effects of essential oils of Turkish oregano (*Origanum minutiflorum*), bay laurel (*Laurus nobilis*), Spanish lavender (*Lavandula stoechas* L.), and fennel (*Foeniculum vulgare*) on common foodborne pathogens. *Journal of Agricultural and Food Chemistry*, *52*(26), 8255−8260.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*, *23*(2), 174−181.

Daglia, M., Cuzzoni, M. T., & Dacarro, C. (1994). Antibacterial activity of coffee: relationship between biological activity and chemical markers. *Journal of Agricultural and Food Chemistry*, *42*(10), 2273−2277.

Dash, S. S., & Gummadi, S. N. (2008). Inhibitory effect of caffeine on growth of various bacterial strains. *Research Journal of Microbiology*, *3*, 457−465.

Davidson, P. M. (2001). Chemical preservatives and naturally antimicrobial compounds. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food microbiology: fundamentals and frontiers* (2nd ed., pp. 593−628). Washington: ASM.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*, *74*(1), 101−109.

Dhanavade, M. J., Jalkute, C. B., Ghosh, J. S., & Sonawane, K. D. (2011). Study antimicrobial activity of Lemon (Citrus lemon L.) peel extract. *British Journal of Pharmacology and Toxicology*, *2*(3), 119−122.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*(2), 308−316.

Elgayyar, M., Draughon, F. A., Golden, D. A., & Mount, J. R. (2001). Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *Journal of Food Protection*, *64*(7), 1019−1024.

Esimone, C., Okoye, F., Nworu, C., & Agubata, C. (2008). In vitro interaction between caffeine and some penicillin antibiotics against *Staphylococcus aureus*. *Tropical Journal of Pharmaceutical Research*, *7*(2), 969−974.

Fisher, K., & Phillips, C. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends in Food Science and Technology*, *19*(3), 156−164.

Gould, S. W. J., Fielder, M. D., Kelly, A. F., & Naughton, D. P. (2009). Anti-microbial activities of pomegranate rind extracts: enhancement by cupric sulphate against clinical isolates of *S. aureus*, MRSA and PVL positive CA-MSSA. *BMC Complementary and Alternative Medicine*, *9*(1), 23.

Griffin, S. G., Markham, J. L., & Leach, D. N. (2000). An agar dilution method for the determination of the minimum inhibitory concentration of essential oils. *Journal of Essential Oil Research*, *12*(2), 249−255.

Gulluce, M., Sahin, F., Sokmen, M., Ozer, H., Daferera, D., Sokmen, A., et al. (2007). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. *Food Chemistry*, *103*(4), 1449−1456.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, *124*(1), 91−97.

Gyawali, R., Adkins, A., Minor, R. C., & Ibrahim, S. A. (2014). Behavior and changes in cell morphology of *Escherichia coli* O157: H7 in liquid medium and skim milk in the presence of caffeine. *CyTA-Journal of Food*, *12*(3), 235−241.

Gyawali, R., & Ibrahim, S. A. (2012). Impact of plant derivatives on the growth of foodborne pathogens and the functionality of probiotics. *Applied Microbiology and Biotechnology*, *95*(1), 29−45.

Gyawali, R., Ibrahim, S. A., Abu Hasfa, S. H., Smqadri, S. Q., & Haik, Y. (2011). Antimicrobial activity of copper alone and in combination with lactic acid against *Escherichia coli* O157: H7 in laboratory medium and on the surface of lettuce and tomatoes. *Journal of Pathogens*, *2011*, 650968.

Hara-Kudo, Y., Kobayashi, A., Sugita-Konishi, Y., & Kondo, K. (2004). Antibacterial activity of plants used in cooking for aroma and taste. *Journal of Food Protection*, *67*(12), 2820−2824.

Hayek, S. A., Gyawali, R., & Ibrahim, S. A. (2013). Antimicrobial natural products. In A. Méndez-Vilas (Ed.), *Microbial pathogens and strategies for combating them: Science, technology and education* (Vol. 2, pp. 910−921). Formatex Research Center.

Hayek, S.A., & Ibrahim, S.A. (2012). Antimicrobial activity of Xoconostle pears (Opuntia-matudae) against *Escherichia coli* O157:H7 in laboratory medium. *International Journal of Microbiolgy*. http://doi:10.1155/2012/368472.

Holloway, A. C., Mueller-Harvey, I., Gould, S. W., Fielder, M. D., Naughton, D. P., & Kelly, A. F. (2012). The effect of copper (ii), iron (ii) sulphate, and vitamin C combinations on the weak antimicrobial activity of (+)-catechin against *Staphylococcus aureus* and other microbes. *Metallomics*, *4*(12), 1280−1286.

Ho, C.-H., Noryati, I., Sulaiman, S.-F., & Rosma, A. (2010). In vitro antibacterial and antioxidant activities of *Orthosiphon stamineus* Benth. extracts against foodborne bacteria. *Food Chemistry*, *122*(4), 1168−1172.

Hoshino, N., Kimura, T., Hayakawa, F., Yamaji, A., & Ando, T. (2000). Bactericidal activity of catechin-copper (II) complexes against *Staphylococcus aureus* compared with *Escherichia coli*. *Letters in Applied Microbiology*, *31*(3), 213−217.

Hsieh, P.-C., Mau, J.-L., & Huang, S.-H. (2001). Antimicrobial effect of various combinations of plant extracts. *Food Microbiology*, *18*(1), 35−43.

Ibrahim, S. A., Bor, T., Song, D., & Tajkarimi, M. (2011). Survival and growth characteristics of *Escherichia coli* O157: H7 in pomegranate carrot and pomegranate-apple blend juices. *Food and Nutrition*, *2*, 844−851.

Ibrahim, S. A., Salameh, M. M., Phetsomphou, S., Yang, H., & Seo, C. W. (2006). Application of caffeine, 1,3,7-trimethylxanthine, to control *Escherichia coli* O157:H7. *Food Chemistry*, *99*(4), 645−650.

Indu, M. N., Hatha, A. A. M., Abirosh, C., Harsha, U., & Vivekanandan, G. (2006). Antimicrobial activity of some of the south-Indian spices against serotypes of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Aeromonas hydrophila*. *Brazilian Journal of Microbiology*, *37*(2), 153−158.

Iu, J., Mittal, G. S., & Griffiths, M. W. (2001). Reduction in levels of *Escherichia coli* O157:H7 in apple cider by pulsed electric fields. *Journal of Food Protection*, *64*, 964−969.

Iwasaki, Y., Hirasawa, T., Maruyama, Y., Ishii, Y., Ito, R., Saito, K., et al. (2011). Effect of interaction between phenolic compounds and copper ion on antioxidant and pro-oxidant activities. *Toxicology In vitro*, *25*(7), 1320−1327.

Karabagias, I., Badeka, A., & Kontominas, M. G. (2011). Shelf life extension of lamb meat using thyme or oregano essential oils and modified atmosphere packaging. *Meat Science*, *88*(1), 109−116.

Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramovič, H., Miloš, M., et al. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chemistry*, *119*(2), 715−723.

Khalid, N., Fawad, S. A., & Ahmed, I. (2011). Antimicrobial activity, phytochemical profile and trace minerals of black mulberry (*Morus nigra* L.) fresh juice. *Pakistan Journal of Botany*, *43*, 91−96.

Kim, E. L., Choi, N. H., Bajpai, V. K., & Kang, S. C. (2008). Synergistic effect of nisin and garlic shoot juice against *Listeria monocytogenes* in milk. *Food Chemistry*, *110*(2), 375−382.

Kim, S.-Y., Kang, D.-H., Kim, J.-K., Ha, Y.-G., Hwang, Ju Y., Kim, T., et al. (2011). Antimicrobial activity of plant extracts against *Salmonella* Typhimurium, *Escherichia coli* O157: H7, and *Listeria monocytogenes* on fresh lettuce. *Journal of Food Science*, *76*(1), M41−M46.

Kim, T. J., Silva, J. L., Weng, W. L., Chen, W. W., Corbitt, M., Jung, Y. S., et al. (2009). Inactivation of *Enterobacter sakazakii* by water-soluble muscadine seed extracts. *International Journal of Food Microbiology*, *129*(3), 295−299.

Kim, T. J., Weng, W. L., Silva, J. L., Jung, Y. S., & Marshall, D. (2010). Identification of natural antimicrobial substances in red muscadine juice against *Cronobacter sakazakii*. *Journal of Food Science*, *75*(3), M150−M154.

Kim, T. J., Weng, W. L., Stojanovic, J., Lu, Y., Jung, Y. S., & Silva, J. L. (2008). Antimicrobial effect of water-soluble muscadine seed extracts on *Escherichia coli* O157: H7. *Journal of Food Protection*, *71*(7), 1465−1468.

Lai, P. K., & Roy, J. (2004). Antimicrobial and chemopreventive properties of herbs and spices. *Current Medicinal Chemistry*, *11*(11), 1451−1460.

Leuschner, R. G. K., & Zamparini, J. (2002). Effects of spices on growth and survival of *Escherichia coli* O157 and *Salmonella enterica* serovar Enteritidis in broth model systems and mayonnaise. *Food Control*, *13*(6), 399−404.

Lewis, K., & Ausubel, F. M. (2006). Prospects for plant-derived antibacterials. *Nature Biotechnology*, *24*(12), 1504−1507.

Lu, Y., & Wu, C. (2010). Reduction of *Salmonella enterica* contamination on grape tomatoes by washing with thyme oil, thymol, and carvacrol as compared with chlorine treatment. *Journal of Food Protection*, *73*(12), 2270−2275.

Magariños, H. L. E., Sahr, C., Selaive, S. D. C., Costa, M. E., Figuerola, F. E., & Pizarro, O. A. (2008). In vitro inhibitory effect of cranberry (*Vaccinium macrocarpum* Ait.) juice on pathogenic microorganisms. *Applied Biochemistry and Microbiology*, *44*(3), 300−304.

Mau, J. L., Chen, C. H., & Hseih, P. C. (2002). The antimicrobial effect of Chinese chive. *Food Science and Agricultural Chemistry*, *4*, 95−103.

Mau, J.-L., Chen, C.-P., & Hsieh, P.-C. (2001). Antimicrobial effect of extracts from Chinese chive, cinnamon, and corni fructus. *Journal of Agricultural and Food Chemistry*, *49*(1), 183−188.

Mo, H., Zhu, Y., & Chen, Z. (2008). Microbial fermented tea−a potential source of natural food preservatives. *Trends in Food Science and Technology*, *19*(3), 124−130.

Moore, K. L., Patel, J., Jaroni, D., Friedman, M., & Ravishankar, S. (2011). Antimicrobial activity of apple, hibiscus, olive, and hydrogen peroxide formulations against *Salmonella enterica* on organic leafy greens. *Journal of Food Protection*, *74*(10), 1676−1683.

Moore-Neibel, K., Gerber, C., Patel, J., Friedman, M., & Ravishankar, S. (2012). Antimicrobial activity of lemongrass oil against Salmonella enterica on organic leafy greens. *Journal of Applied Microbiology*, *112*(3), 485−492.

Munoz, M., Guevara, L., Palop, A., Tabera, J., & Fernández, P. S. (2009). Determination of the effect of plant essential oils obtained by supercritical fluid extraction on the growth and viability of *Listeria monocytogenes* in broth and food systems using flow cytometry. *LWT−Food Science and Technology*, *42*(1), 220−227.

Nguyen, P., & Mittal, G. S. (2007). Inactivation of naturally occurring microorganisms in tomato juice using pulsed electric field (PEF) with and without antimicrobials. *Chemical Engineering and Processing: Process Intensification*, *46*(4), 360−365.

Orue, N., García, S., Feng, P., & Heredia, N. (2013). Decontamination of *Salmonella*, *Shigella*, and *Escherichia coli* O157:H7 from leafy Green vegetables using edible plant extracts. *Journal of Food Science*, *78*(2). M290−M296.

Özcan, M. (2004). Mineral contents of some plants used as condiments in Turkey. *Food Chemistry*, *84*(3), 437−440.

Özkan, G., Sagdiç, O., Göktürk Baydar, N., & Kurumahmutoglu, Z. (2004). Antibacterial activities and total phenolic contents of grape pomace extracts. *Journal of the Science of Food and Agriculture*, *84*(14), 1807−1811.

Park, S.-H., Choi, Mi-R., Park, J.-W., Park, Ki-H., Chung, M.-S., Ryu, S., et al. (2011). Use of organic acids to inactivate *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science*, *76*(6), M293−M298.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—potential applications in food safety and quality. *Food Research International*, *44*(4), 827−839.

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., & Oksman-Caldentey, K. M. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, *90*(4), 494−507.

Qiu, X., & Wu, V. C. H. (2007). Evaluation of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* Typhimurium and *Staphylococcus aureus* in ground beef with cranberry concentrate by thin agar layer method. *Journal of Rapid Methods and Automation in Microbiology*, *15*(3), 282−294.

Ramanavičienė, A., Mostovojus, V., Bachmatova, I., & Ramanavičius, A. (2003). Anti-bacterial effect of caffeine on *Escherichia coli* and *Pseudomonas fluorescens*. *Acta Medica Lituanica*, *10*(4), 185−188.

Razavi Rohani, S. M., Moradi, M., Mehdizadeh, T., Saei-Dehkordi, S. S., & Griffiths, M. W. (2011). The effect of nisin and garlic (*Allium sativum* L.) essential oil separately and in combination on the growth of *Listeria monocytogenes*. *LWT−Food Science and Technology*, *44*(10), 2260−2265.

Rhodes, P. L., Mitchell, J. W., Wilson, M. W., & Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from Vitis vinifera variety Ribier. *International Journal of Food Microbiology*, *107*(3), 281−286.

Ruban, P., & Gajalakshmi, K. (2012). In vitro antibacterial activity of Hibiscus rosa−sinensis flower extract against human pathogens. *Asian Pacific Journal of Tropical Biomedicine*, *2*(5), 399−403.

Sagdic, O., Kuscu, A., Ozcan, M., & Ozcelik, S. (2002). Effects of Turkish spice extracts at various concentrations on the growth of *Escherichia coli* O157:H7. *Food Microbiology*, *19*(5), 473−480.

Sakanaka, S., Juneja, L. R., & Taniguchi, M. (2000). Antimicrobial effects of green tea polyphenols on thermophilic spore-forming bacteria. *Journal of Bioscience and Bioengineering*, *90*(1), 81−85.

Shan, B., Cai, Yi-Z., Brooks, J. D., & Corke, H. (2007). The in vitro antibacterial activity of dietary spice and medicinal herb extracts. *International Journal of Food Microbiology*, *117*(1), 112−119.

Silván, J. M., Mingo, E., Hidalgo, M., de Pascual-Teresa, S., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2013). Antibacterial activity of a grape seed extract and its fractions against *Campylobacter* spp. *Food Control*, *29*(1), 25−31.

Singh, A., Singh, R. K., Bhunia, A. K., & Singh, N. (2003). Efficacy of plant essential oils as antimicrobial agents against *Listeria monocytogenes* in hotdogs. *LWT−Food Science and Technology*, *36*(8), 787−794.

Singh, B., Bernadette Falahee, M., & Adams, M. R. (2001). Synergistic inhibition of *Listeria monocytogenes* by nisin and garlic extract. *Food Microbiology*, *18*(2), 133−139.

Sivasankaran-Nair, M., & Selwin-Joseyphus, R. (2008). Synthesis and characterization of Co(II), Ni(II), Cu(II) and Zn(II) complexes of tridentate Schiff base derived from vanillin and DL-α-aminobutyric acid. *Spectrochim Acta*, *70*, 749−753.

Sofos, J. N., & Geornaras, I. (2010). Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Science*, *86*(1), 2−14.

Sreeramulu, G., Zhu, Y., & Knol, W. (2000). Kombucha fermentation and its antimicrobial activity. *Journal of Agricultural and Food Chemistry*, *48*(6), 2589−2594.

Stewart, G. S. A. B., Jassim, S. A. A., Denyer, S. P., Newby, P., Linley, K., & Dhir, V. K. (1998). The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *Journal of Applied Microbiology*, *84*(5), 777−783.

Su, X., Howell, A. B., & D'Souza, D. H. (2012). Antibacterial effects of plant-derived extracts on methicillin-resistant *Staphylococcus aureus*. *Foodborne Pathogens and Disease*, *9*(6), 573−578.

Svoboda, K. P., & Greenaway, R. I. (2003). Lemon scented plants. *International Journal of Aromatherapy*, *13*(1), 23−32.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, *21*(9), 1199−1218.

Tassou, C., Koutsoumanis, K., & Nychas, G.-J. E. (2000). Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Research International*, *33*(3), 273−280.

Thongson, C., Davidson, P. M., Mahakarnchanakul, W., & Vibulsresth, P. (2005). Antimicrobial effect of Thai spices against *Listeria monocytogenes* and *Salmonella* Typhimurium DT104. *Journal of Food Protection*, *68*(10), 2054−2058.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987−6000.

Ulusoy, S., Boşgelmez-Tinaz, G., & Seçilmiş-Canbay, H. (2009). Tocopherol, carotene, phenolic contents and antibacterial properties of rose essential oil, hydrosol and absolute. *Current Microbiology*, *59*(5), 545−558.

Wallace, R. J. (2004). Antimicrobial properties of plant secondary metabolites. *Proceedings of the Nutrition Society*, *63*(04), 621−629.

Wan, J., Wilcock, A., & Coventry, M. J. (1998). The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *Journal of Applied Microbiology*, *84*(2), 152−158.

Weerakkody, N. S., Caffin, N., Turner, M. S., & Dykes, G. A. (2010). In vitro antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bacteria. *Food Control*, *21*(10), 1408−1414.

Xi, Y., Sullivan, G. A., Jackson, A. L., Zhou, G. H., & Sebranek, J. G. (2011). Use of natural antimicrobials to improve the control of *Listeria monocytogenes* in a cured cooked meat model system. *Meat Science*, *88*(3), 503−511.

Xu, X., Yan, M., & Zhu, Y. (2005). Influence of fungal fermentation on the development of volatile compounds in the Puer tea manufacturing process. *Engineering in Life Sciences*, *5*(4), 382−386.

Yadav, S., Gite, S., Nilegaonkar, S., & Agte, V. (2011). Effect of supplementation of micronutrients and phytochemicals to fructooligosaccharides on growth response of probiotics and *E. coli*. *Biofactors*, *37*(1), 58−64.

Yuste, J., & Fung, D. Y. C. (2002). Inactivation of *Listeria monocytogenes* Scott A 49594 in apple juice supplemented with cinnamon. *Journal of Food Protection*, *65*(10), 1663−1666.

Zaika, L. L. (1988). Spices and herbs: their antimicrobial activity and its determination. *Journal of Food Safety*, *9*(2), 97−118.

Zaika, L. L., & Kissinger, J. C. (1984). Fermentation enhancement by spices: identification of active component. *Journal of Food Science*, *49*(1), 5−9.

Plant extracts as antimicrobials in food products: mechanisms of action, extraction methods, and applications

3

R. Gyawali, S.A. Hayek, S.A. Ibrahim
North Carolina A&T State University, Greensboro, NC, USA

3.1 Introduction

Plants contain an array of natural compounds with antimicrobial activity. In recent years, there has been an increased interest in natural antimicrobials, especially those obtained from plants. For example, a number of published authors have reported the antimicrobial effect of plant extracts obtained from spices, herbs, fruits, and vegetables (Burt, 2004; Davidson, 1997; Hayek, Gyawali, & Ibrahim, 2013; Tajkarimi, Ibrahim, & Cliver, 2010; Tiwari et al., 2009). However, the exact mechanism of action of these antimicrobial products is not well documented. The response of Gram-positive bacteria to plant extracts is generally much greater than that of Gram-negative bacteria. This is mainly due to the presence of lipopolysaccharide cell wall in Gram-negative bacteria that prevents the diffusion of hydrophobic compounds. On the other hand, the growth in antibiotic resistance in pathogenic microorganisms has turned the research toward plant extracts as potential alternatives. Plant extracts seem to be a promising solution to the increasing antibiotic resistance, and may also provide better results than synthetic preservatives (Hayek et al., 2013). In addition, plant extracts have been shown to reduce antibiotic resistance by promoting synergistic effects between natural antimicrobials and antibiotics.

The extraction method plays an important role in the overall effect of natural antimicrobial products. Active compounds have been extracted and purified for research or other applications using different extraction methods. The extraction process usually involves the use of chemical solvents or heat, which could affect the functionality of active compounds in plants. We have proposed a novel and effective approach, known as the *direct extraction method*. Direct extraction is a chemical solvent-free and heat-free method that depends mainly on mechanical extraction of products; therefore, this method could avoid possible alteration of the natural composition of plant extracts or their active components. This chapter summarizes the mechanisms of action of plant extracts as antimicrobials, their effectiveness in

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reducing antibiotic resistance in pathogens, method of extraction, antimicrobial activity against Gram-positive and Gram-negative bacteria, and potential applications in food products.

3.2 Mechanisms of action of plant extracts

The exact mechanism(s) or target(s) of natural antimicrobials are often unknown or not fully understood. However, different modes of action for different antimicrobial groups have been reviewed. Since it is difficult to identify a specific action site where many interactions take place simultaneously, different versions of proposed mechanisms of action have been reported (Davidson, 1997; Gyawali & Ibrahim, 2012; Holley & Patel, 2005). Some possible modes of actions are listed below. Table 3.1 lists some of the commonly used plant species/compounds and their modes of antimicrobial action on target pathogens.

Potential mechanisms of action include the following:

- Membrane-disrupting compounds could cause leakage of cellular content, interference with active transport or metabolic enzymes, or dissipation of cellular energy in ATP form (Davidson, 1997).
- Direct pH reduction of the substrate or growth medium due to an increase in proton concentration, depression of the internal cellular pH by ionization of the undissociated acid molecule, or disruption of substrate transport by alteration of cell membrane permeability (Davidson, 1997).
- Organic acids present in plant species may also inhibit NADH oxidation, thus eliminating supplies of reducing agents to electron transport systems (Davidson, 1997). The undissociated portion of an acid molecule is primarily responsible for the antimicrobial activity; effectiveness at a given pH depends largely on the dissociation constant (pK_a) of the acid (Hosein, Breidt, & Smith, 2011; Olasupo, Fitzgerald, Narbad, & Gasson, 2004).
- Organic acids interfere with membrane permeability. Short-chain organic acids interfere with energy metabolism by altering the structure of the cytoplasmic membrane through interaction with membrane proteins (Ricke, 2003).
- Essential oils (EOs) produce structural and functional damage to the bacterial cell membrane, where optimal range of hydrophobicity is also involved in the toxicity of EOs (Goni et al., 2009).
- Antimicrobial compounds in plants can attack the phospholipid bilayer of the cell membrane, disrupt the cell enzyme systems, compromise the genetic material of a bacterial cell, or form fatty acid hydroperoxidase by oxygenation of unsaturated fatty acids (Burt et al., 2007).
- The antimicrobial activity of flavonoids is largely due to the ability of these compounds to penetrate the cell membrane of bacteria (Davidson & Naidu, 2000). Membrane damage and changes in intercellular pH, membrane potential and adenosine triphosphate synthesis (Sanchez, García, & Heredia, 2010).

The above-mentioned possible modes of action are the most familiar mechanisms used to explain the antimicrobial effect of natural products, including plant extracts. However, the exact antimicrobial mechanisms of action of specific natural compounds against the target microorganisms are still unknown. The specific plant compound or substance may act differently for a particular group of target pathogens, as listed

Table 3.1 Proposed antibacterial mechanisms of selected plant-derived extracts

Plant extracts/compound	Microorganisms	Mode of action	References
Tea tree oil	*Escherichia coli*, *Staphylococcus aureus*	Disrupt cytoplasmic membrane	Cox et al. (1998)
Essential oils (EOs) of oregano, savory, lemongrass, and active compounds such as thymol, eugenol, and carvacrol	*E. coli*, *E. coli* O157:H7, *Listeria monocytogenes*, *Lactobacillus sakei*, *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, and *S. aureus*	Disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP	Lambert, Skandamis, Coote, and Nychas (2001), Gill and Holley (2006), Oussalah, Caillet, and Lacroix (2006), and Raybaudi-Massilia, Mosqueda-Melgar, and Martin-Belloso (2006)
Cinnamon oil and cinnamaldehyde	*E. coli*, *E. coli* O157:H7, and *L. monocytogenes*	Decrease in the intracellular ATP by ATPase activity without apparent changes on the cell membrane	Gill and Holley (2004, 2006) and Oussalah et al. (2006)
Carvacrol and thymol	*E. coli* and *Salmonella* Typhimurium	Disintegration of outer membrane	Helander et al. (1998)
Thyme EO	*L. monocytogenes*	Thickening, disruption of the cell wall, increased roughness, and lack of cytoplasm	Rasooli, Rezaei, and Allameh (2006)
Citrus EO	*Enterococcus* spp.	Morphological changes	Fisher and Phillips (2008)
Grape seed extract	*Methicillin-resistant* S. aureus	Reduced residual cellular content with partial disintegration of the bacterial cell surfaces	Al-Habib, Al-Saleh, Safer, and Afzal (2010)

Continued

Table 3.1 Continued

Plant extracts/compound	Microorganisms	Mode of action	References
Cranberry extract	*S. aureus*	Cell wall damages and impaired cells surrounded by lysate material	Wu, Qiu, Bushway, and Harper (2008)
Grape seed and pomegranate polyphenols	*S. aureus*	Cell surface roughening and formation of blobs on cell surface	Su, Howell, and D'Souza (2012)
Garlic extract	*Salmonella Hadar*	Loss of enzymatic activities, rupture cell wall, and nonhomogeneous disposition of cytoplasmic material	Belguith et al. (2009)
Coriander oil	*Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311, *S. aureus* ATCC 25923, *S. aureus* (SA08)	Bacterial cell permeabilization. As a result of membrane permeabilization, all other cellular functions such as membrane potential, respiratory activity, or efflux pump activity are also compromised	Silva, Ferreira, Queiroz, and Domingues (2011)
Lemongrass EO	*Salmonella* Enteritidis	Disruption of cell membrane and leakage of cell content	Raybaudi-Massilia et al. (2006).

in Table 3.1. Thus, the type of damage observed could be different among bacteria from one to another even when treated with the same antimicrobial substance. In summary, the death of the bacterium can occur mainly by (Juven, Kanner, Schved, & Weisslowicz, 1994; Shimamura, Zhao, & Hu, 2007):

1. Physical disruption of the membrane
2. Dissipation of the proton motive force
3. Inhibition of membrane-associated enzyme activity

3.3 Plant extracts and antibiotic resistance

Antibiotics are proven effective treatments for microbial diseases. However, recently there has been a rise in microorganisms that are resistant against common antibiotic drugs; in fact, antibiotic-resistant bacteria are emerging rapidly (Palaniappan & Holley, 2010). These drug-resistant bacteria are increasing due to the resistivity that bacteria have developed and lack of new antimicrobials to combat them. Efficient antimicrobials need to kill bacterial cells quickly to avoid building antibiotic resistance in the bacteria. Plant-derived antimicrobial agents could be effective and safe alternatives to fight against antibiotic-resistant pathogens, especially those pathogens associated with foodborne illnesses.

Recent research has demonstrated that as bacteria become more resistant to antibiotics, traditional food preservation methods of drying, heat, and acid treatment may not be effective (Álvarez-Ordóñez, Fernández, Bernardo, & López, 2009; Mani-López, García, & López-Malo, 2012). Moreover, intensive use of chemical preservatives in foods and other agricultural commodities has contributed significantly to antibiotic resistance; many of these chemical preservatives have caused increased consumer concerns about their safety, emphasizing the need for new antimicrobial agents that are safe and effective. Plant-derived compounds could be used in the development of new preservation systems; thus, these compounds could prove to be among the most promising solutions for microbial resistance (Hayek et al., 2013). Moreover, natural compounds could meet the increasing consumer demand for healthier foods. Plant extracts could also be used to combat infections caused by multidrug-resistant bacteria. Extracts from plants could be used in combination with antibiotics to restore the efficacy of these drugs for resistant pathogenic bacteria (Abreu, McBain, & Simões, 2012).

Phytochemicals in plant extracts can reduce antibiotic resistance of bacteria (Abreu et al., 2012). Thymol and carvacrol are highly effective in reducing the resistance of *Salmonella* Typhimurium SGI 1 (*tet* A) to ampicillin, tetracycline, penicillin, bacitracin, erythromycin, and novobiocin, and reducing the resistance of *S. pyogene*s with the resistant gene ermB to erythromycin (Palaniappan & Holley, 2010). Thymol and cinnamaldehyde are effective in reducing the MICs of ampicillin, tetracycline, penicillin, erythromycin, and novobiocin with *Escherichia coli* N00-666. Similarly, carvacrol, thymol, and cinnamaldehyde were effective against *Staphylococcus aureus (S. aureus)*, having the resistant gene blaZ, and they reduced the MICs of ampicillin, penicillin, and bacitracin. These results indicate that plant extracts, when used in

combination with antibiotics, can decrease the MICs of antibiotics in drug-resistant microorganisms (Palaniappan & Holley, 2010). Aqueous extracts of tea (*Camellia sinensis*) can also reduce the MICs of high-level *methicillin-resistant* S. aureus (MRSA) (256 μg/ml) to less than 0.12 μg/ml for methicillin and penicillin (Stapleton et al., 2004). Esimone, Okoye, Nworu, and Agubata (2008) reported the antibacterial effectiveness of amoxicillin in combination with caffeine, suggesting that caffeine could also enhance the effectiveness of antibiotics.

Livermore (2000) reported that staphylococci are generally susceptible to most antibiotics; however, there are frequent causes of morbidity as well as mortality due to the ability of staphylococci to develop resistance through both mutation and the transfer of DNA between bacteria (Scheffler, Colmer, Tynan, Demain, & Gullo, 2013). Braga et al. (2005) reported the antimicrobial effect of pomegranate extract on antibiotic-sensitive and -resistant strains of *S. aureus*. The authors reported the synergistic effect of pomegranate extract (0.1−2.0% (*v*/*v*)) with chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin, indicating the potential for plant extracts to enhance the activity of these antibiotics. Because plant extracts kill bacteria or inhibit their growth, plant products could become important ingredients in new antimicrobial drugs to combat antibiotic-resistant microorganisms (Clatworthy, Pierson, & Hung, 2007). Extracts or phytochemicals found in plant components act via a different mechanism than that of antibiotics (Abreu et al., 2012).

3.4 Extraction methods to maximize antimicrobial properties

Most common methods of plant extraction are time consuming and cumbersome. In addition, most of the extraction methods require large amounts of different solvents, and some of these solvents (hydrochloric acid, ammonium chloride, ethanol, methanol, and alcohol) are considered toxic (Herrero, Cifuentes, & Ibanez, 2006). Common extraction procedures, including chemical or heat treatments, can also alter the active ingredients' total content, functionality, or natural characteristics, or could produce unsafe compounds (Beta, Rooney, Marovatsanga, & Taylor, 2000; Hayek et al., 2013; Lin & Chou, 2009). In addition, the extraction of EOs is sometimes expensive and requires several complex steps (Conte, Speranza, Sinigaglia, & Del Nobile, 2007; Lambert et al., 2001). Thus, an extraction method with minimal processing, such as juice or mechanical direct extraction, seems to be more promising to avoid possible alteration or destruction of active ingredients. In this section, we have highlighted the idea of using direct, aqueous, or juice extraction methods to minimize both costs and possible alteration to active compounds. The extract obtained by these methods can be used directly as an antimicrobial agent against foodborne pathogens.

Over the last 10 years, our research group has developed a simple and new extraction method called *direct extraction*, which is rapid and does not require the use of chemical solvents. Direct extraction can be implemented without the need for any special or advanced technology. This method is simply based on collecting the liquid portion of the natural material and testing them directly against microorganisms,

and has proven to be quite effective in controlling the growth of foodborne pathogens (Hayek & Ibrahim, 2012; Ibrahim, Bor, Song, & Tajkarimi, 2011; Ibrahim, Tse, Yang, & Fraser, 2009; Ibrahim, Yang, Song, & Tse, 2011). Figure 3.1 shows a schematic diagram of the proposed extraction method. Direct extraction is simple and cost effective, and can be applied in the laboratory without the need for advanced technology. However, when the direct extract is not sufficient enough to cause microbial growth inhibition, the extract can be further concentrated with ultrafiltration methods and later applied as an antimicrobial agent. Ultrafiltration techniques remove the aqueous phase and lead to an increased amount of antimicrobial compounds in the final extract solution, which could lead to a stronger antimicrobial effect. The ultrafiltration technique has limited practical applications in plant extract concentration, while it is widely used in the dairy industry for preconcentration of milk.

Aqueous plant extracts have been proposed as alternatives to chemical extract methods to reduce contamination of foods without changing the food properties (Valtierra-Rodriguez, Heredia, Garcia, & Sanchez, 2010). Plant species contain various water-soluble antimicrobial active compounds that could be extracted by using water as a solvent or directly used as a pure extract. Typically, to be considered a 100% natural compound, plant products should be extracted with water as a solvent. Plant products such as leaves, bark, seeds, and roots can either be soaked in warm water or boiled in the water. The extracts can be sterile filtered and applied as antimicrobial agents. The use of water may cause dilution in the active ingredient concentration and make it less effective. Active compounds can be reconcentrated using ultrafiltration.

Juice from fruits and vegetables can also be directly extracted for use as antimicrobials. Vegetables such as carrots and cabbage and their juices have naturally occurring antimicrobials, and have shown inhibitory effects against pathogenic microorganisms. It has been reported that carrot cells have antilisterial activity due to the presence of antimicrobials or phytoalexins in the carrot's cellular and vascular fluids. Similarly, cabbage juice contains antimicrobial thiosulfinates (Scollard, Francis, & O'Beirne, 2013).

The proposed methods—direct, aqueous, and juice extraction—have been used widely to study the antimicrobial activity of plant extracts. For example, pressed vegetable juices (Chinese chive, cabbage, white purslane, and bamboo shoot) have been shown to inhibit the growth of common foodborne microorganisms including bacteria, yeasts, and molds (Mau, Chen, & Hsieh, 2001). Aloe vera juice showed antimicrobial activity against *Mycobacterium smegmatis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Micrococcus luteus*, *Candida albicans*, and *Bacillus sphericus* using the disc diffusion method (30 μl/disc) (Alemdar & Agaogla, 2009). Aloe vera gel has been used in the food industry as a functional ingredient in drinks, beverages, and ice creams. Valverde et al. (2005) reported the use of aloe vera gel as an edible coating in fruits, which would be an innovative and interesting means of commercial application and an alternative to the use of postharvest chemical treatments. The antimicrobial activity of water extract from garlic showed inhibition of *E. coli* and *Salmonella* Typhi (Arora & Kaur, 1999). Sakagami, Kaikoh, Kajimura, and Yokoyama (2000) investigated the effect of clove extract on the production of verotoxins by enterohemorrhagic (EHEC) *E. coli* O157:H7. Water-distilled extract of clove at a concentration of 0.5%

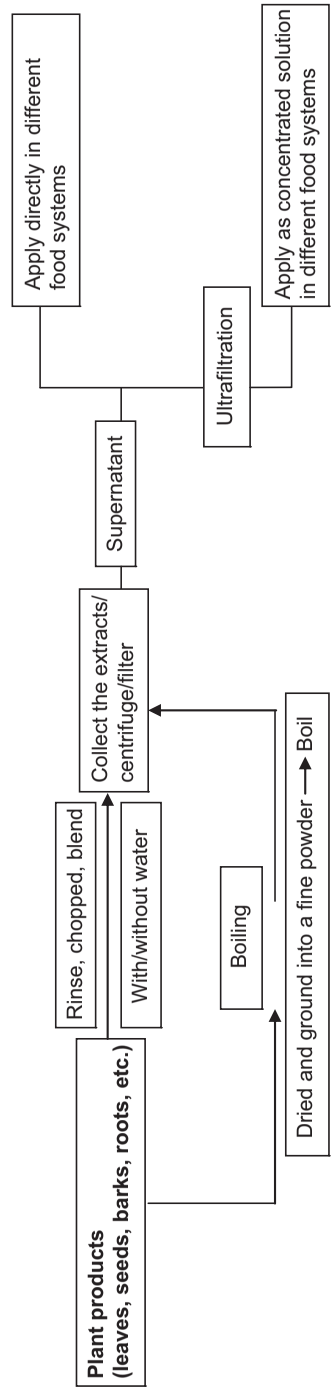

Figure 3.1 Schematic diagram showing the proposed extraction method.

(*w*/*v*) was shown to inhibit the production of EHEC verotoxin. Belguith et al. (2009) reported the use of aqueous garlic extracts obtained by simple steps (blend, centrifuged, and filter sterilized). It was reported that extract at 11−13 mg/ml had a bacteriostatic effect on *Salmonella Hadar*. Heat treatment of water-soluble muscadine seed extracts can enhance the antimicrobial activity by increasing acidity, total phenolics, and individual phenolic compounds (Kim et al., 2008). Thus, direct extract or juice extraction seem to be the most promising methods to avoid possible alteration or modification for the nature of natural antimicrobial compounds.

Direct extraction has been used in our laboratory for 10 years, showing consistent results. Our research group has studied the effects of several plant species extracts against various foodborne pathogens. The direct extract from Chinese chive is an effective plant extract that can inhibit a wide range of microorganisms (Hsieh, Mau, & Huang, 2001; Ibrahim et al., 2009). Ibrahim et al. (2009) showed total growth inhibition with 500 μl of chive direct extract against *Salmonella* spp. over 24 h of incubation. Direct extract of xoconostle pears was tested against four strains of *E. coli* O157:H7 in brain heart infusion (BHI) laboratory medium using growth over time and agar well diffusion assays. Xoconostle pears (*Opuntia matudae*) had a significant ($P < 0.05$) inhibitory effect at 4, 6, and 8% (*v*/*v*) concentrations and complete inhibitory effect at 10% (*v*/*v*) during 8 h of incubation at 37 °C (Hayek & Ibrahim, 2012). Ibrahim, Yang, et al. (2011b) demonstrated the antimicrobial activity of guava extract (5%) against *E. coli* O157:H7 and *Salmonella* in liquid medium. In the presence of guava extract, the bacterial population remained at 3.0 log CFU/ml as initial inoculum level, while the control sample population reached 7.0−8.0 log CFU/ml. This result demonstrated the potential of guava extract as an effective antimicrobial agent in food safety. Similarly, the addition of pomegranate juice inhibited the growth of tested *E. coli* O157:H7 in both laboratory medium and blended juices (carrot and apple juice). The bacterial population was reduced by less than at least 2 log CFU/ml in BHI broth and juice blend samples in the presence of 20% and 40% pomegranate juice, respectively (Ibrahim, Bor, et al., 2011a).

3.5 Response of Gram-positive and Gram-negative bacteria to plant extracts

Gram-positive bacteria are more susceptible to EOs and various plant extractions. Plant EOs appeared more active with respect to Gram reaction, thereby exerting a greater inhibitory effect against Gram-positive bacteria than Gram-negative bacteria, which are more resistant to the EOs (Deans, Noble, Hiltunen, Wuryani, & Penzes, 1995). Yoda, Hu, Zhao, and Shimamura (2004) investigated the antibacterial activity of tea catechin (epigallocatechin gallate) against various strains of *Staphylococcus* (Gram-positive cocci) and *E. coli*, *K. pneumonia*, and *Salmonella* (Gram-negative rods). The results of Yoda et al. (2004) showed that 50−100 μg/ml is required to inhibit the growth of *Staphylococcus*, whereas concentrations higher than 800 μg/ml are required to inhibit Gram-negative rods. In similar work, *S. aureus* was the most sensitive and *E. coli* was the most resistant against various extracts of dietary spices

(100 mg/ml) when these bacteria were tested using the agar well diffusion method (Shan, Cai, Brooks, & Corke, 2007). Belguith et al. (2009) also reported the impact of aqueous garlic extract mainly on Gram-negative bacteria, and their outer membrane, which is absent in Gram-positive bacteria. It was found from *in vitro* experiments that citrus oils (lemon, orange, and bergamot) are more effective on Gram-positive bacteria (*Listeria monocytogenes*, *S. aureus*, *Bacillus cereus*) than on Gram-negative (*E. coli* O157 and *Campylobacter jejuni)* (Burt, 2004; Delaquis, Stanich, Girard, & Mazza, 2002; Fisher & Phillips, 2006). Earlier, Shelef, Naglik, and Bogen (1980) and Tassou, Drosinos, and Nychas (1995) reported that Gram-positive bacteria are more sensitive to EOs than Gram-negative bacteria. Thongson, Davidson, Mahakarnchanakul, and Weiss (2004) reported higher MIC values for *Salmonella* Typhimurium than *L. monocytogenes*, indicating the greater sensitivity of Gram-positive listeriae to spice extracts than the Gram-negative salmonellae. Commonly, Gram-negative bacteria are more resistant to plant oils due to the presence of their hydrophilic cell wall structure. Gram-negative bacteria contain a lipopolysaccharide that blocks the penetration of hydrophobic oil and avoids the accumulation of EOs in target cell membranes (Bezic, Skočibušić, Dunkić, & Radonić, 2003; Zaika, 1988). Davidson and Branen (2005) also suggested that the outer membrane surrounding the cell walls of Gram-negative bacteria may restrict the diffusion of hydrophobic compounds through the bacterium's lipopolysaccharide cell wall coverings. This could explain why Gram-positive bacteria are more sensitive to plant extracts than Gram-negative bacteria (Rahman & Kang, 2009).

3.6 Applications of plant extracts in food products

Although limited research has been conducted on plant extracts and their EOs used as antimicrobials in the food industry, it has been shown that these extracts have potential antimicrobial properties against foodborne pathogens. Antimicrobial agents from plants have been mostly used in the form of biofilm and edible coatings in food systems. These antimicrobial films and coatings slowly diffuse inside the food package, thus extending the duration of the antimicrobial activity. Ouattara, Simard, Piette, Begin, and Holley (2000) reported an increased shelf life for shrimp when thyme oil and trans-cinnamaldehyde were added to the shrimp's soy and whey protein-derived coating. Ouattara and Mafu (2003) reported a 20–21-day increased shelf life for shrimp by using a combination of a protein-based coating containing 0.9% thyme oil and 1.8% *trans*-cinnamaldehyde with 3.0 kGy gamma irradiation. The use of rosemary and thyme extract in combination with irradiation extended the microbiological shelf life of chicken at 4 °C by seven to eight times compared to untreated chicken (Mahrour, Lacroix, Nketsa-Tabiri, Calderon, & Gagnon, 1998). The antilisterial effects of salad dressings, oil, and lemon juice or vinegar (with or without prior microwave oven heating), on frankfurters during simulated home storage was evaluated (Shen, Georgnaras, Kendall, & Sofos, 2009). The authors reported a significant reduction of *L. monocytogenes* for frankfurters dipped in salad dressing in combination with oil and lemon juice or vinegar as compared to those dipped in distilled water. The

population of *L. monocytogenes* was reduced when chicken frankfurters were treated with clove oil at 1 or 2% and stored at 5 °C for 2 weeks or 15 °C for 1 week, but survived and grew on control frankfurters (Mytle, Anderson, Doyle, & Smith, 2006). Valtierra-Rodriguez et al. (2010) demonstrated that the extract of lime alone or in combination with other edible fruits reduced the population of *C. jejuni* and *Campylobacter coli* in chicken skin by >4.0 log cycles. When spices were added to a complex food system such as ground beef, the inhibitory activity of these spices decreased significantly (Uhart, Maks, & Ravishankar, 2006). Similarly, when chive extract was added to food samples, *Salmonella* populations were reduced to 1.78 and 2.10 log CFU/ml in chicken and beef broth, respectively. In the control samples, *Salmonella* populations reached 7.40 log CFU/ml in chicken broth and 8.67 log CFU/ml in beef broth. The findings showed that crude chive extract can be effective against the growth of *Salmonella* and could be used in food products to prevent the growth of this pathogen (Ibrahim et al., 2009). Careaga et al. (2003) showed the inhibitory effect of bell pepper (*Capsicum annum*) extract against *Salmonella* Typhimurium in raw beef. The authors reported 1.5 ml/100 g of meat as the minimum concentration of the extract to prevent the growth of *Salmonella* Typhimurium.

Green tea extract has been used in various food applications such as bread (Wang & Zhou, 2004), extra virgin olive oil (Rosenblat, Volkova, Coleman, Almagor, & Aviram, 2008), meat, fish (Alghazeer, Saeed, & Howell, 2008), dehydrated apple products (Lavelli, Vantaggi, Corey, & Kerr, 2010), rice starch products (Wu, Chen, Li, & Li, 2009), and biscuits (Mildner-Szkudlarz, Zawirska-Wojtasiak, Obuchowski, & Gośliński, 2009). Grape seed extract has also demonstrated antimicrobial activities alone or in combination with other hurdle technologies in various food applications such as tomatoes, frankfurters, raw and cooked meat, poultry products, and fish (Bisha, Weinsetel, Brehm-Stecher, & Mendonca, 2010; Brannan, 2009; Perumalla & Hettiarachchy, 2011). The combined effect of polyphenol-rich cocoa powder with pulsed electric field significantly inactivated the level of *Cronobacter sakazakii* in infant milk formula over 12 h at 8 °C storage. In this study, a reduction of 4.41 log cycles was achieved when 12% of cocoa powder was added 4 h after the pulsed electric field application (Pina-Pérez, Martínez-López, & Rodrigo, 2012). Recently Gyawali, Adkins, Minor, and Ibrahim (2014) demonstrated antimicrobial effect of caffeine against *E. coli* O157:H7 in skim milk. Authors have shown potential application of caffeine as a preservative in beverages.

Plant extracts or EOs are more effective in culture medium (laboratory medium) than in food systems. Table 3.2 compares the antimicrobial efficacy of plant extracts in laboratory medium and food systems. Burt (2004) also reported that a higher concentration of EOs is needed to achieve the same effect in food experiments as *in vitro* (growth media). Two-fold differences of EOs in semiskim milk, 10-fold in pork liver sausage, 50-fold in soup, and 25−100-fold in soft cheese is required to get the same efficacy as *in vitro* experiments (Burt, 2004). In addition, poor water solubility of most EOs extracted from thyme, oregano, and clove makes it difficult to incorporate EOs into complex food systems, hence reduces the antimicrobial activity (Ananda Baskaran, Kazmer, Hinckley, Andrew, & Venkitanarayanan, 2009; Friedman, Henika, Levin, & Mandrell, 2004; Gaysinsky, Taylor, Davidson, Bruce, & Weiss, 2007).

Table 3.2 Comparing the antimicrobial efficacy of selected plant extracts in laboratory medium and food systems

Antimicrobial effect comparison				
Plant components/ products	**Pathogens**	**Laboratory medium**	**Food systems**	**References**
Arrowroot tea extracts	*Escherichia coli* O157: H7, *Salmonella* Enteritidis, *Listeria monocytogenes, Staphylococcus aureus*	5% of extract showed 6−7 log suppression of growth at 35 °C in 3 days	Populations of *Salmonella* and *Listeria* suppressed by only 1.5 log cycles at 7 °C on day 7 at 3% and 6% extract	Kim and Fung (2004a, 2004b)
Essential oils (EOs) of thyme, clove, and pimento	*L. monocytogenes*	Highly effective in peptone water	Efficacy of EOs was reduced in food due to interaction with food components	Singh, Singh, Bhunia, and Singh (2003)
Filipendula ulmaria extract	*E. coli* O157:H7 NCTC 12900, *Salmonella* Enteritidis PT4, *L. monocytogenes* Scott A, *S. aureus* ATCC 6538, *Pseudomonas fragi* ATCC 4973, *Shewanella putrefaciens* ATCC 8071, *Yersinia enterocolitica* CITY 844, *Aeromonas caviae* 27994 INCO, and *Brochothrix thermosphacta* BR1	Showed antimicrobial activity in the presence of lactic acid	Antimicrobial activity was limited in the tested real food systems	Boziaris, Proestos, Kapsokefalou, and Komaitis (2011)

Fresh garlic paste	*E. coli* O157:H7	Decrease of 1.27 log CFU/g in buffered peptone water at 2 weeks at 8 °C	Did not show any antimicrobial activity in ground beef	Gupta and Ravishankar (2005)
Garlic, ginger, turmeric paste	*Salmonella* Typhimurium DT 104	1.0−1.5 log Cycle reduction in buffered peptone water stored at 4 and 8 °C for 10 days	Slight decrease (<1 log) in *Salmonella* population observed in ground beef	Uhart et al. (2006)
Mint EO (0·5, 1·0, 1·5, 2·0%, *v/w*)	*Salmonella* Enteritidis and *L. monocytogenes*	No growth was observed over 2 days at 30 °C	*Salmonella* Enteritidis died in food tzatziki (pH 4.5), survived in food pâté (pH 6·8) at 10 °C	Tassou et al. (1995)
Tea extracts	*E. coli* O157:H7, *Salmonella enterica* serovar Enteritidis, *L. monocytogenes,* and *S. aureus*	Approximately 3.0−5.0 log cycle suppression of *L. monocytogenes and S. aureus*	Population of *L. monocytogenes* and *S. aureus* in ground beef were not significantly different than the control	Kim, Ruengwilysup, and Fung (2004)
Thyme EO (0.6%)	*L. monocytogenes*	No growth within the incubation period of 32 h	About 1.0 log cycle reduction in beef stored at 4 °C for 12 days	Solomakos, Govaris, Koidis, and Botsoglou (2008)

Gill, Delaquis, Russo, and Holley (2002) reported that the greater availability of nutrients in foods than in laboratory media may enable bacteria to repair damaged cells faster. The bacteria's protection against the action of these natural extracts is attributed to the high levels of fat and/or protein in the foodstuffs (Burt, 2004).

Moreover, higher levels of these EOs can affect the original sensory properties. It is therefore important to consider the organoleptic effects, and to assess whether the use of high amounts of these natural preservatives can alter the taste of food (Nazer, Kobilinsky, Tholozan, & Dubois-Brissonnet, 2005). Conversely, lower concentrations of these extracts in combination with other natural preservatives may lower the negative sensory effect.

3.7 Conclusion

With today's consumers demanding alternatives to synthetic antimicrobials for food application, plant extracts or plant-derived compounds could potentially provide the ideal solution. Natural antimicrobials can also be used to increase the antibiotic susceptibility of drug-resistant bacteria. Our established method of using direct extracts from plant compounds (without any chemical processing) as antimicrobial agents would serve to further validate the use of the word "natural." These extracts can be used either directly after extraction or after ultrafiltration. Future work is also needed to investigate the effect of natural plant extracts in combination with other food processing techniques.

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References

Abreu, A. C., McBain, A. J., & Simões, M. (2012). Plants as sources of new antimicrobials and resistance-modifying agents. *Natural Product Reports*, *29*(9), 1007−1021.

Alemdar, S., & Agaoglu, S. (2009). Investigation of in vitro antimicrobial activity of aloe vera juice. *Journal of Animal and Veterinary Advances*, *8*(1), 99−102.

Al-Habib, A., Al-Saleh, E., Safer, A.-M., & Afzal, M. (2010). Bactericidal effect of grape seed extract on methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Toxicological Sciences*, *35*(3), 357−364.

Alghazeer, R., Saeed, S., & Howell, N. K. (2008). Aldehyde formation in frozen mackerel (*Scomber scombrus*) in the presence and absence of instant green tea. *Food Chemistry*, *108*(3), 801−810.

Álvarez-Ordóñez, A., Fernández, A., Bernardo, A., & López, M. (2009). Comparison of acids on the induction of an acid tolerance response in *Salmonella* Typhimurium, consequences for food safety. *Meat Science*, *81*, 65−70.

Ananda Baskaran, S., Kazmer, G. W., Hinckley, L., Andrew, S. M., & Venkitanarayanan, K. (2009). Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro. *Journal of Dairy Science*, *92*(4), 1423−1429.

Arora, D. S., & Kaur, J. (1999). Antimicrobial activity of spices. *International Journal of Antimicrobial Agents*, *12*(3), 257−262.

Belguith, H., Kthiri, F., Ben Ammar, A., Jaafoura, H., Ben Hamida, J., & Landoulsi, A. (2009). Morphological and biochemical changes of *Salmonella hadar* exposed to aqueous garlic extract. *International Journal of Morphology*, *27*, 705−713.

Beta, T., Rooney, L. W., Marovatsanga, L. T., & Taylor, J. R. N. (2000). Effect of chemical treatments on polyphenols and malt quality in sorghum. *Journal of Cereal Science*, *31*(3), 295−302.

Bezić, N., Skočibušić, M., Dunkić, V., & Radonić, A. (2003). Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. *Phytotherapy Research*, *17*(9), 1037−1040.

Bisha, B., Weinsetel, N., Brehm-Stecher, B. F., & Mendonca, A. (2010). Antilisterial effects of gravinol-s grape seed extract at low levels in aqueous media and its potential application as a produce wash. *Journal of Food Protection*, *73*(2), 266−273.

Boziaris, I. S., Proestos, C., Kapsokefalou, M., & Komaitis, M. (2011). Antimicrobial effect of *Filipendula ulmaria* plant extract against selected foodborne pathogenic and spoilage bacteria in laboratory media, fish flesh and fish roe product. *Food Technology and Biotechnology*, *49*(2), 263−270.

Braga, L. C., Leite, A. A. M., Xavier, K. G. S., Takahashi, J. A., Bemquerer, M. P., Chartone-Souza, E., et al. (2005). Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus*. *Canadian Journal of Microbiology*, *51*(7), 541−547.

Brannan, R. G. (2009). Effect of grape seed extract on descriptive sensory analysis of ground chicken during refrigerated storage. *Meat Science*, *81*(4), 589−595.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223−253.

Burt, S. A., van der Zee, R., Koets, Ad P., de Graaff, A. M., van Knapen, F., Gaastra, W., et al. (2007). Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157: H7. *Applied and Environmental Microbiology*, *73*(14), 4484−4490.

Careaga, M., Fernández, E., Dorantes, L., Mota, L., Jaramillo, M. E., & Hernandez-Sanchez, H. (2003). Antibacterial activity of *Capsicum* extract against *Salmonella* Typhimurium and *Pseudomonas aeruginosa* inoculated in raw beef meat. *International Journal of Food Microbiology*, *83*(3), 331−335.

Clatworthy, A. E., Pierson, E., & Hung, D. T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology*, *3*(9), 541−548.

Conte, A., Speranza, B., Sinigaglia, M., & Del Nobile, M. A. (2007). Effect of lemon extract on foodborne microorganisms. *Journal of Food Protection*, *70*(8), 1896−1900.

Cox, S. D., Gustafson, J. E., Mann, C. M., Markham, J. L., Liew, Y. C., Hartland, R. P., et al. (1998). Tea tree oil causes K+ leakage and inhibits respiration in *Escherichia coli*. *Letters in Applied Microbiology*, *26*(5), 355−358.

Davidson, P. M. (1997). Chemical preservatives and natural antimicrobial compounds. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food microbiology: fundamentals and frontiers* (pp. 520−556). Washington, DC: American Society for Microbiology.

Davidson, P. M., & Branen, A. L. (2005). Food antimicrobials − an introduction. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobial in food* (3rd ed.). (pp. 1−10). New York, NY: CRC Press.

Davidson, P. M., & Naidu, A. S. (2000). Phyto-phenols. In A. S. Naidu (Ed.), *Natural food antimicrobial systems* (pp. 265−294). Boca Raton, FL: CRC Press.

Deans, S. G., Noble, R. C., Hiltunen, R., Wuryani, W., & Penzes, L. G. (1995). Antimicrobial and antioxidant properties of *Syzygium aromaticum* (L.) Merr. & Perry: impact upon bacteria, fungi and fatty acid levels in ageing mice. *Flavour and Fragrance Journal*, *10*(5), 323−328.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*, *74*(1), 101−109.

Esimone, C., Okoye, F., Nworu, C., & Agubata, C. (2008). In vitro interaction between caffeine and some penicillin antibiotics against *Staphylococcus aureus*. *Tropical Journal of Pharmaceutical Research*, *7*(2), 969−974.

Fisher, K., & Phillips, C. A. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni, Escherichia coli* O157, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems. *Journal of Applied Microbiology*, *101*(6), 1232−1240.

Fisher, K., & Phillips, C. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends in Food Science and Technology*, *19*(3), 156−164.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2004). Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *Journal of Agricultural and Food Chemistry*, *52*(19), 6042−6048.

Gaysinsky, S., Taylor, T. M., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157: H7. *Journal of Food Protection*, *70*(11), 2631−2637.

Gill, A. O., Delaquis, P., Russo, P., & Holley, R. A. (2002). Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *International Journal of Food Microbiology*, *73*(1), 83−92.

Gill, A. O., & Holley, R. A. (2004). Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Applied and Environmental Microbiology*, *70*(10), 5750−5755.

Gill, A. O., & Holley, R. A. (2006). Disruption of *Escherichia coli, Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology*, *108*(1), 1−9.

Goni, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., & Nerín, C. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chemistry*, *116*(4), 982−989.

Gupta, S., & Ravishankar, S. (2005). A comparison of the antimicrobial activity of garlic, ginger, carrot, and turmeric pastes against *Escherichia coli* O157:H7 in laboratory buffer and ground beef. *Foodborne Pathogens and Disease*, *2*(4), 330−340.

Gyawali, R., Adkins, A., Minor, R. C., & Ibrahim, S. A. (2014). Behavior and changes in cell morphology of *Escherichia coli* O157:H7 in liquid medium and skim milk in the presence of caffeine. *CyTA-Journal of Food*, *12*(3), 235−241. http://dx.doi.org/10.1080/19476337.2013.834977.

Gyawali, R., & Ibrahim, S. A. (2012). Impact of plant derivatives on the growth of foodborne pathogens and the functionality of probiotics. *Applied Microbiology and Biotechnology*, *95*(1), 29−45.

Hayek, S. A., Gyawali, R., & Ibrahim, S. A. (2013). Antimicrobial natural products. In A. Méndez-Vilas (Ed.), *Microbial pathogens and strategies for combating them: Science, technology and education* (Vol. 2, pp. 910−921). Formatex Research Center.

Hayek, S. A., & Ibrahim, S. A. (2012). Antimicrobial activity of xoconostle pears (*Opuntia matudae*) against *Escherichia coli* O157: H7 in laboratory medium. *International Journal of Microbiology*, *2012*, (Article ID 368472), 1−6. http://dx.doi.org/10.1155/2012/368472.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., et al. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *Journal of Agricultural and Food Chemistry*, *46*(9), 3590−3595.

Herrero, M., Cifuentes, A., & Ibanez, E. (2006). Sub-and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae: a review. *Food Chemistry*, *98*(1), 136−148.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*, *22*(4), 273−292.

Hosein, A. M., Breidt, F., & Smith, C. E. (2011). Modeling the effects of sodium chloride, acetic acid, and intracellular pH on survival of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, *77*(3), 889−895.

Hsieh, P.-C., Mau, J.-L., & Huang, S.-H. (2001). Antimicrobial effect of various combinations of plant extracts. *Food Microbiology*, *18*(1), 35−43.

Ibrahim, S. A., Bor, T., Song, D., & Tajkarimi, M. (2011). Survival and growth characteristics of *Escherichia coli* O157: H7 in pomegranate-carrot and pomegranate-apple blend juices. *Food and Nutrition*, *2*, 844−851.

Ibrahim, S., Tse, T., Yang, H., & Fraser, A. (2009). Antibacterial activity of a crude chive extract against *Salmonella* in culture medium, beef broth and chicken broth. *Food Protection Trends*, *29*(3), 155−160.

Ibrahim, S. A., Yang, G., Song, D., & Tse, T. S. (2011). Antimicrobial effect of guava on *Escherichia coli* O157: H7 and *Salmonella* Typhimurium in liquid medium. *International Journal of Food Properties*, *14*(1), 102−109.

Juven, B. J., Kanner, J., Schved, F., & Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Microbiology*, *76*(6), 626−631.

Kim, S., & Fung, D. Y. C. (2004a). Antibacterial effect of crude water-soluble arrowroot (Puerariae radix) tea extracts on food-borne pathogens in liquid medium. *Letters in Applied Microbiology*, *39*(4), 319−325.

Kim, S., & Fung, D. Y. C. (2004b). Antibacterial effect of water-soluble arrowroot (Puerariae radix) tea extracts on foodborne pathogens in ground beef and mushroom soup. *Journal of Food Protection*, *67*(9), 1953−1956.

Kim, S., Ruengwilysup, C., & Fung, D. Y. C. (2004). Antibacterial effect of water-soluble tea extracts on foodborne pathogens in laboratory medium and in a food model. *Journal of Food Protection*, *67*(11), 2608−2612.

Kim, T., Weng, W., Stojanovic, J., Lu, Y., Jung, Y., & Silva, J. (2008). Antimicrobial effect of water-soluble muscadine seed extracts on *Escherichia coli* O157: H7. *Journal of Food Protection*, *71*, 1465.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J., & Nychas, G.-J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, *91*(3), 453−462.

Lavelli, V., Vantaggi, C., Corey, M., & Kerr, W. (2010). Formulation of a dry green tea-apple product: study on antioxidant and color stability. *Journal of Food Science*, *75*(2), C184−C190.

Lin, Y. C., & Chou, C. C. (2009). Effect of heat treatment on total phenolic and anthocyanin contents as well as antioxidant activity of the extract from *Aspergillus awamori*-fermented black soybeans, a healthy food ingredient. *International Journal of Food Sciences and Nutrition*, *60*(7), 627−636.

Livermore, D. M. (2000). Antibiotic resistance in staphylococci. *International Journal of Antimicrobial Agents*, *16*, 3−10.

Mahrour, A., Lacroix, M., Nketsa-Tabiri, J., Calderon, N., & Gagnon, M. (1998). Antimicrobial properties of natural substances in irradiated fresh poultry. *Radiation Physics and Chemistry*, *52*(1), 81−84.

Mani-López, E., García, H. S., & López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, *45*(2), 713−721.

Mau, J.-L., Chen, C.-P., & Hsieh, P.-C. (2001). Antimicrobial effect of extracts from Chinese chive, cinnamon, and corni fructus. *Journal of Agricultural and Food Chemistry*, *49*(1), 183−188.

Mildner-Szkudlarz, S., Zawirska-Wojtasiak, R., Obuchowski, W., & Gośliński, M. (2009). Evaluation of antioxidant activity of green tea extract and its effect on the biscuits lipid fraction oxidative stability. *Journal of Food Science*, *74*(8), S362−S370.

Mytle, N., Anderson, G. L., Doyle, M. P., & Smith, M. A. (2006). Antimicrobial activity of clove (*Syzgium aromaticum*) oil in inhibiting *Listeria monocytogenes* on chicken frankfurters. *Food Control*, *17*(2), 102−107.

Nazer, A. I., Kobilinsky, A., Tholozan, J.-L., & Dubois-Brissonnet, F. (2005). Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect? *Food Microbiology*, *22*(5), 391−398.

Olasupo, N., Fitzgerald, D., Narbad, A., & Gasson, M. (2004). Inhibition of *Bacillus subtilis* and *Listeria innocua* by nisin in combination with some naturally occurring organic compounds. *Journal of Food Protection*, *67*(3), 596−600.

Ouattara, B., & Mafu, A. A. (2003). Natural antimicrobials in combination with gamma irradiation. In S. MéRoller (Ed.), *Natural antimicrobials for the minimal processing of foods* (pp. 263−271). Boca Raton, Fla.: CRC Press.

Ouattara, B., Simard, R. E., Piette, G., Begin, A., & Holley, R. A. (2000). Diffusion of acetic and propionic acids from chitosan-based antimicrobial packaging films. *Journal of Food Science*, *65*(5), 768−773.

Oussalah, M., Caillet, S., & Lacroix, M. (2006). Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157: H7 and *Listeria monocytogenes*. *Journal of Food Protection*, *69*(5), 1046−1055.

Palaniappan, K., & Holley, R. A. (2010). Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *International Journal of Food Microbiology*, *140*(2), 164−168.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—potential applications in food safety and quality. *Food Research International*, *44*(4), 827−839.

Pina-Pérez, M. C., Martínez-López, A., & Rodrigo, D. (2012). Cocoa powder as a natural ingredient revealing an enhancing effect to inactivate *Cronobacter sakazakii* cells treated by pulsed electric Fields in infant milk formula. *Food Control*, *32*(1), 87−92.

Rahman, A., & SunChul, K. (2009). In vitro control of food-borne and food spoilage bacteria by essential oil and ethanol extracts of *Lonicera japonica* Thunb. *Food Chemistry*, *116*(3), 670−675.

Rasooli, I., Rezaei, M. B., & Allameh, A. (2006). Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes*. *International Journal of Infectious Diseases*, *10*(3), 236−241.

Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., & Martin-Belloso, O. (2006). Antimicrobial activity of essential oils on *Salmonella* Enteritidis, *Escherichia coli*, and *Listeria innocua* in fruit juices. *Journal of Food Protection*, *69*(7), 1579−1586.

Ricke, S. C. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science*, *82*(4), 632−639.

Rosenblat, M., Volkova, N., Coleman, R., Almagor, Y., & Aviram, M. (2008). Anti-atherogenicity of extra virgin olive oil and its enrichment with green tea polyphenols in the atherosclerotic apolipoprotein-E-deficient mice: enhanced macrophage cholesterol efflux. *The Journal of Nutritional Biochemistry*, *19*(8), 514−523.

Sakagami, Y., Kaikoh, S., Kajimura, K., & Yokoyama, H. (2000). Inhibitory effect of clove extract on vero-toxin production by enterohemorrhagic *Escherichia coli* O157:H7. *Biocontrol Science*, *5*(1), 47−49.

Sánchez, E., García, S., & Heredia, N. (2010). Extracts of edible and medicinal plants damage membranes of *Vibrio cholerae*. *Applied and Environmental Microbiology*, *76*(20), 6888−6894.

Scheffler, R. J., Colmer, S., Tynan, H., Demain, A. L., & Gullo, V. P. (2013). Antimicrobials, drug discovery, and genome mining. *Applied Microbiology and Biotechnology*, *97*(3), 969−978.

Scollard, J., Francis, G. A., & O'Beirne, D. (2013). Some conventional and latent anti-listerial effects of essential oils, herbs, carrot and cabbage in fresh-cut vegetable systems. *Postharvest Biology and Technology*, *77*, 87−93.

Shan, B., Cai, Yi-Z., Brooks, J. D., & Corke, H. (2007). The in vitro antibacterial activity of dietary spice and medicinal herb extracts. *International Journal of Food Microbiology*, *117*(1), 112−119.

Shelef, L. A., Naglik, O. A., & Bogen, D. W. (1980). Sensitivity of some common food-borne bacteria to the spices sage, rosemary, and allspice. *Journal of Food Science*, *45*(4), 1042−1044.

Shen, C., Geornaras, I., Kendall, P. A., & Sofos, J. N. (2009). Antilisterial activities of salad dressings, without or with prior microwave oven heating, on frankfurters during simulated home storage. *International Journal of Food Microbiology*, *132*(1), 9−13.

Shimamura, T., Zhao, W.-H., & Hu, Z.-Q. (2007). Mechanism of action and potential for use of tea catechin as an antiinfective agent. *Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents)*, *6*(1), 57−62.

Silva, F., Ferreira, S., Queiroz, J. A., & Domingues, F. C. (2011). Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. *Journal of Medical Microbiology*, *60*(10), 1479−1486.

Singh, A., Singh, R. K., Bhunia, A. K., & Singh, N. (2003). Efficacy of plant essential oils as antimicrobial agents against *Listeria monocytogenes* in hotdogs. *LWT-Food Science and Technology*, *36*(8), 787−794.

Solomakos, N., Govaris, A., Koidis, P., & Botsoglou, N. (2008). The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. *Food Microbiology*, *25*(1), 120−127.

Stapleton, P. D., Shah, S., Anderson, J. C., Hara, Y., Hamilton-Miller, J. M. T., & Taylor, P. W. (2004). Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *International Journal of Antimicrobial Agents*, *23*, 462−467.

Su, X., Howell, A. B., & D'Souza, D. H. (2012). Antibacterial effects of plant-derived extracts on methicillin-resistant *Staphylococcus aureus*. *Foodborne Pathogens and Disease*, *9*(6), 573−578.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, *21*(9), 1199−1218.

Tassou, C. C., Drosinos, E. H., & Nychas, G. J. E. (1995). Effects of essential oil from mint (*Mentha piperita*) on *Salmonella* Enteritidis and *Listeria monocytogenes* in model food systems at 4 and 10 °C. *Journal of Applied Microbiology*, *78*(6), 593−600.

Thongson, C., Davidson, P. M., Mahakarnchanakul, W., & Weiss, J. (2004). Antimicrobial activity of ultrasound-assisted solvent-extracted spices. *Letters in Applied Microbiology*, *39*(5), 401−406.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987−6000.

Uhart, M., Maks, N., & Ravishankar, S. (2006). Effect of spices on growth and survival of *Salmonella* Typhimurium DT 104 in ground beef stored at 4 and 8 °C. *Journal of Food Safety*, *26*(2), 115−125.

Valtierra-Rodriguez, D., Heredia, N. L., Garcia, S., & Sanchez, E. (2010). Reduction of *Campylobacter jejuni* and *Campylobacter coli* in poultry skin by fruit extracts. *Journal of Food Protection*, *73*(3), 477−482.

Valverde, J. M., Valero, D., Martínez-Romero, D., Guillén, F., Castillo, S., & Serrano, M. (2005). Novel edible coating based on aloe vera gel to maintain table grape quality and safety. *Journal of Agricultural and Food Chemistry*, *53*(20), 7807−7813.

Wang, R., & Zhou, W. (2004). Stability of tea catechins in the breadmaking process. *Journal of Agricultural and Food Chemistry*, *52*(26), 8224−8229.

Wu, Y., Chen, Z., Li, X., & Li, M. (2009). Effect of tea polyphenols on the retrogradation of rice starch. *Food Research International*, *42*(2), 221−225.

Wu, V. C. H., Qiu, X., Bushway, A., & Harper, L. (2008). Antibacterial effects of American cranberry *(Vaccinium macrocarpon)* concentrate on foodborne pathogens. *LWT-Food Science and Technology*, *41*(10), 1834−1841.

Yoda, Y., Hu, Z.-Q., Zhao, W.-H., & Shimamura, T. (2004). Different susceptibilities of *Staphylococcus* and Gram-negative rods to epigallocatechin gallate. *Journal of Infection and Chemotherapy*, *10*(1), 55−58.

Zaika, L. L. (1988). Spices and herbs: their antimicrobial activity and its determination. *Journal of Food Safety*, *9*(2), 97−118.

Bacteriophages as antimicrobials in food products: history, biology and application

4

H. Anany[1,2], *L.Y. Brovko*[1], *T. El-Arabi*[2], *M.W. Griffiths*[1]
[1]University of Guelph, Guelph, ON, Canada; [2]Ain Shams University, Cairo, Egypt

4.1 Introduction

Globalisation of the food supply and consumer demand for wholesome, minimally processed and organic food has driven the search for novel strategies to provide safer food with longer shelf life, which avoid the use of chemical preservatives and excessive physical treatments that affect organoleptic and nutritional quality of food products. Natural biopreservatives, such as antimicrobial compounds of animal or plant origin as well as antagonistic microorganisms, have been used for centuries. For example, herbs and spices have been added to food, not only to add flavour but also to preserve food. Foods have also been fermented and pickled to extend shelf life. Renewed interest has been focused on natural antimicrobials as an alternative to 'chemical' preservatives to improve safety, enhance hygienic quality and extend shelf life of foods (Juneja, Dwivedi, & Yan, 2012), largely because of consumer perceptions that 'synthetic' chemicals are bad for you.

Generally natural antimicrobials can be divided into two major categories. The first is comprised of compounds isolated from animals, plants or microorganisms with pronounced antibacterial properties against a wide range of bacterial pathogens and food-spoilage organisms. These include essential oils, bacteriocins and enzymes/proteins such as lysozyme, lactoferrin, protamine and endolysins. The second category is made up of living organisms including antagonistic bacteria (Galvez, Abriouel, Benomar, & Lucas, 2010) and bacteriophages (Coffey, Mills, Coffey, Mcauliffe, & Ross, 2010; Garcia, Rodriguez, Rodriguez, & Martinez, 2010). Of these two approaches, the latter may offer an advantage, as evolution has endowed these organisms with the ability to adapt to various environments, allowing them to exert their natural antibacterial properties in complex matrices such as food. Furthermore, both bacteria and bacteriophages are easy to propagate, which reduces the cost of their production. Bacteriophages (phages) as antimicrobials provide another favourable feature – they target specific bacteria, and hence provide an opportunity to tailor the treatment towards the selected bacterium and/or group of bacteria. This feature may be especially important for production of foods that involve fermentation, as a pathogen-specific phage will not inactivate the starter cultures responsible for the fermentation. Bacteriophages are abundant in the environment, and we consume them daily in large numbers with food and drink. They are considered as safe, and some bacteriophages are listed

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by US Food and Drug Administration (FDA) as GRAS (Generally Recognised As Safe), which allows producers to use them as processing aids to control *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat meats or raw red meats (Sillankorva, Oliveira, & Azeredo, 2012).

4.2 Research into bacteriophages

Bacteriophages – obligatory parasites infecting bacteria (bacterial viruses) – were co-discovered (probably independently) by the English microbiologist Frederick Twort and the French-Canadian Felix d'Herelle between 1915 and 1920 following the observation that very small filterable particles were able to kill bacteria (Duckworth, 1976). This feature of bacteriophages immediately attracted the attention of researchers who were looking for efficient cures for various infectious diseases, and their interest was further piqued as these antimicrobial agents were several thousand-fold more potent than any other agent known at that time (Fruciano & Bourne, 2007). Multiple trials were carried out worldwide, which confirmed the efficacy of phage application both as therapeutic and prophylactic agents. It was noted, for example, that when traditional interventions (vaccination and water disinfection with potassium permanganate) were used to control cholera outbreaks in India from 1933 to 1935, the average length of the outbreak was 26 days, whereas when bacteriophages were introduced into the water supply, the outbreak length was significantly reduced to 48 h. The successful application of bacteriophage-mediated antimicrobial therapy continued even after the discovery of penicillin. Research at St Mary's Hospital in London, UK, showed that the combined application of penicillin with *Staphylococcus* bacteriophage K produced more rapid killing of the staphylococci than either agent applied separately (Himmelweit, 1945). Nevertheless, the research on medical applications of bacteriophages was practically abandoned during the 1940s in North America and Western Europe for both scientific and social reasons. Lack of sufficient knowledge of bacteriophage biology and physiology prevented elucidation of the mechanism behind bacteriophage-mediated bacterial killing. Arguably, the discovery of penicillin was the greatest contributor to the decline of interest in bacteriophages as antimicrobial agents.

However, basic research on bacteriophage structure, biology and physiology continued despite the reduced interest in their medicinal and prophylactic applications. The nucleoprotein composition of bacteriophages was generally accepted in the early 1930s. However, the origin of bacteriophages (or 'd'Herelle phenomena', as they were termed) remained a subject of controversy. One group of researchers (including d'Herelle himself) considered bacteriophages as a separate living organism (the exogenous concept), and others considered them to be a product of the bacterium (the endogenous concept). Note that neither electron microscopy nor radioactive tracers were available at that time. Significant progress in understanding the nature and physiology (origin) of bacteriophages was achieved by the so-called Phage Group led by the German theoretical physicist Max Delbrück (Summers, 1993). His interest in biological systems, especially in heredity, started in 1931 while working with atomic physicist Nils

Bohr in Copenhagen. In 1937 Delbrück received a Rockefeller fellowship to work in the United States on a very widely defined topic, the 'Mathematical-Physical Investigation of Tissues, Cells, and Molecules' (Vanhelvoort, 1992) and chose bacteriophages as his model organism mainly because they were the simplest to work with, especially for physicists. Together with Emory Ellis they clearly accepted d'Herelle's assertion that bacteriophages were obligate intracellular parasites. By developing the so-called 'one-step growth technique', they managed to show with accurate, statistically valid measurements that the process of bacteriophage growth was limited to a single cycle that included absorption of the phage on the bacterium; the 'latent period', during which the bacteriophage particles were formed within the bacterial cell; and the 'burst' step, when the newly formed phage particles were released into the environment by the dissolution (lysis) of the bacterium. Later these processes were visualised by electron microscopy (Pennazio, 2006). At the same time Delbrück found that bacteriophages could cause bacterial lysis by two different mechanisms: 'lysis from within' (the mechanism described in the one-step growth cycle) and 'lysis from without', which is the result of adsorption of multiple bacteriophages to the same bacterial cell. Delbrück also explained the phenomenon of lysogeny, which is the seemingly spontaneous ability of some bacteria (growing in the absence of phages) to undergo lysis and subsequently produce bacteriophages. These progeny phages are then able to lyse later generations of bacteria, which was a cornerstone in the endogenous theory of bacteriophage origin. He hypothesised that bacterial cells produced a constituent, termed *b*, that was synthesised continuously but in the presence of phage it triggered phage replication. In phage-susceptible bacteria the turnover of *b* in the presence of phage was faster than in cells not exposed to phage, which resulted in cell lysis. However, in bacterial cells that underwent lysogenic conversion, the production of *b* was faster than in cells exposed to phage, and as a result both the bacterium and the phage could replicate without lysis occurring (Vanhelvoort, 1992). Thus, the link between phage multiplication and bacterial metabolism was established. Eventually this hypothesis was proven to be essentially true, and helped lay the foundations of modern molecular biology.

4.3 Biology of bacteriophages

After almost 100 years of research, many hundreds of bacteriophages have been isolated and characterised with varying morphology, size and nucleic acid and protein composition. It is estimated that more than 10^{31} bacteriophages exist in our planet; making them the most abundant and yet most unstudied form of life (Kutter & Sulakvelidze, 2005). Bacteriophages have an average size of 20−200 nm and possess two main components: genetic material in the form of DNA or RNA as a core and a surrounding proteinaceous shell (capsid). It is generally accepted that the function of the protein capsid is to protect the phage genome and to facilitate specific adsorption of the phage to the host cell, which initiates the infection process. Examples of the major morphological types discovered so far and used for bacteriophage classification are presented in Figure 4.1

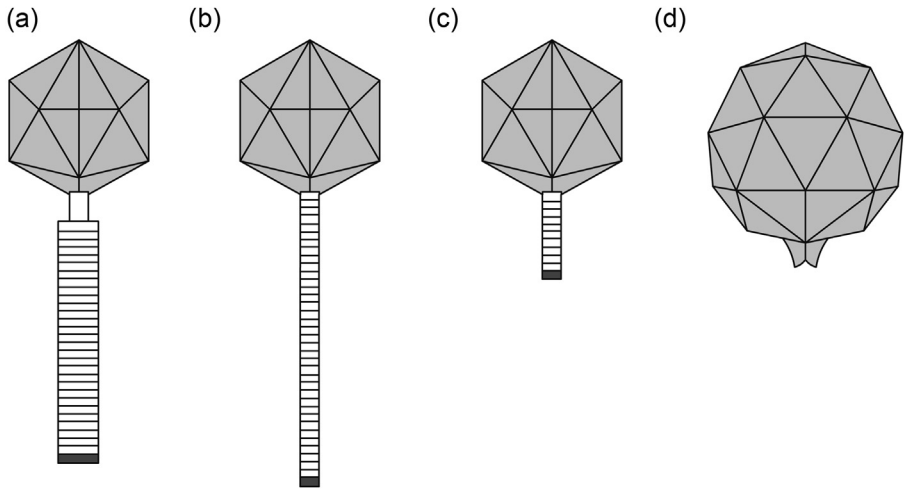

Figure 4.1 Schematic representation of most common phage morphologies (not to scale). (a) Myoviridae − non-enveloped virus with ~50−100 nm diameter head and contractile tail of 80−130 nm long and 15−20 nm diameter. (b) Siphoviridae − non-enveloped virus with 50−65 nm diameter head and long (up to 500 nm) non-contractile tail of 8−10 nm diameter. (c) Podoviridae − non-enveloped virus with 50−65 nm diameter head and short (up to 20 nm) thick tail. (d) Liviviridae − small non-enveloped viruses of 25−30 nm diameter, require bacterial pili to attach to and infect cells.

(Ackermann & Prangishvili, 2012). The majority (96.2%) of the phages investigated by electron microscopy belongs to the tailed types (Ackermann, 2007). The tail usually comprises a hollow tube surrounded in some cases with a contractile sheath that helps injection of nucleic acid into the host bacterium. Bacteriophages are relatively resistant to adverse environmental conditions and they have been found in desert sand and hot springs (Jończyk, Klak, Miedzybrodzki, & Górski, 2011; Prigent, Leroy, Confalonieri, Dutertre, & Dubow, 2005). However, they usually exist in environments with a pH range from 5 to 9 and a temperature below 60 °C.

The current, generally accepted view of the bacteriophage life-cycle, which is applicable to numerous morphologically different phages, is presented schematically in Figure 4.2. Bacteriophages interact with susceptible bacterial cells through receptors located on their tail fibers (in the case of myoviridae, siphoviridae and podoviridae bacteriophages) or on the phage capsid (in the case of leviviridae bacteriophages). Sometimes co-factors are required for the phage receptors to assume the configuration necessary for attachment. L-tryptophan, Ca^{2+} and Mg^{2+} have been identified as co-factors (Anderson, 1992). Phage nucleic acid is very densely packed inside the phage head, creating a high internal pressure in the capsid of approximately 50 atm (Nurmemmedov, Castelnovo, Catalano, & Evilevitch, 2007). This internal pressure is a driving force for the injection of the capsid content into the cell through the formed pores/channels. In the presence of an excess of phage particles over the number of bacterial cells (high multiplicity of infection, MOI), the number of pores formed in the

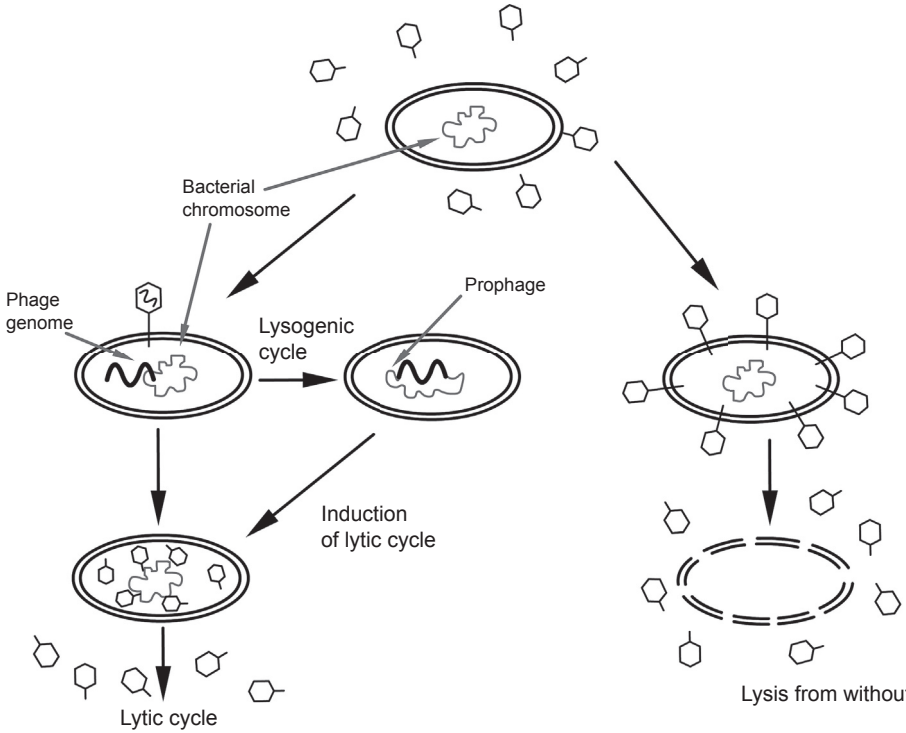

Figure 4.2 Schematic representation of bacteriophage life-cycles.

bacterial cell wall reaches the level at which it cannot maintain turgor pressure and the cell loses its integrity, resulting in the loss of intracellular metabolites and, ultimately, cell death. This mechanism of phage-mediated cell lysis is called 'lysis from without'. In this case bacterial cell lysis is not accompanied by phage replication.

On the other hand, when the MOI is at the level that allows single phage particles to infect the host cell, injection of the phage genome almost immediately initiates a cascade of molecular events. From this point in time two infection scenarios (cycles) may develop depending on both bacteriophage and host cell properties and conditions (Guttman, Raya, & Kutter, 2005).

The bacteriophages termed virulent (lytic) mostly exhibit the so-called lytic cycle, which involves immediate transition from the metabolism of the 'healthy' bacterium to phage-directed metabolism. Various biosynthetic processes of the cell are terminated and replaced by processes directed towards the synthesis of bacteriophage components. The progeny phages are assembled within the infected cell, which is followed by the enzymatic disruption of the cell wall from inside and release of newly formed phage particles into the environment. In all, the process takes about 1 h.

The lysogenic (or temperate) bacteriophages form prophages following injection of their DNA/RNA into the bacterial cell. The phage genome in this case is physically inserted into the host genome by means of specific enzymes, termed integrases. The

bacterium-carrying prophage (lysogen) can continue to divide indefinitely until certain external factors (such as ultraviolet light, presence of antibiotic, etc.) induce the lytic process.

The choice between the two life-cycles is probably made by the bacteriophage based on the presence of sufficient energy in the host cell to make a large burst of phage; if the energy level is low, the formation of prophage offers the better chance for survival.

The accumulated knowledge on basic bacteriophage structure and physiology revived interest in bacteriophages as natural antimicrobial agents at the beginning of this century. Both therapeutic and environmental applications are being investigated. Some examples of applications of bacteriophages for control of bacterial pathogens in food and food-related environments are presented below.

4.4 Bacteriophages as biocontrol agents in food

Even with the widespread introduction of food safety management systems, such as hazard analysis and critical control points (HACCP), throughout the food chain, serious food-borne illness outbreaks and food recalls still occur, along with the emergence of new food-borne pathogens. Recent estimates are that food-borne illness costs the US economy $\$77.7 \times 10^9$ US Dollars/year (Scharff, 2012), while in Canada it costs around CAD$1.33 billion a year (Snowdon, Buzby, & Roberts, 2002). Thus, it is crucial to develop highly robust approaches to mitigate the consequences of these outbreaks and control these different food-borne pathogens during food production, processing and retailing. The available data in the literature on using phages as biocontrol agents to reduce pathogenic bacteria at different stages of food production, both preharvest and postharvest, has shown promise (Coffey et al., 2010; Hagens & Loessner, 2010; McIntyre, Hudson, Billington, & Withers, 2012). Lytic phages have been used successfully to specifically eradicate zoonotic pathogens from living animals, decontaminate carcass meat or the surfaces of ready-to-eat products (Atterbury, 2009; Brovko, Anany, & Griffiths, 2012; Greer, 2005; Hagens & Loessner, 2007). Moreover, the recent regulatory recognition of phage-based commercial lytic products by different government agencies as a safe technique that could be used to enhance food safety (Goodridge & Bisha, 2011) has also given credence to the application of these natural bacterial killers to mitigate the effect of different food-borne pathogens. Table 4.1 presents a list of companies providing commercial phage-based products for biocontrol in the food industry. Based on the information posted on these companies' websites, products that are recognisized by the FDA as GRAS (Generally Recognised As Safe) are identified in this table.

There are several factors of concern when screening phages for use as biocontrol agents. To allow for maximum efficacy, only robust lytic phages should be considered for food applications. Moreover, isolated phages should be screened for their host range and selected based on their ability to effectively infect a broad range of pathogenic strains of their hosts (Hagens & Loessner, 2010). In addition, it is recommended that a cocktail of multiple phages be used to maximise the host range and reduce the

Table 4.1 Examples for commercially available phage-based products to enhance food safety

Company	Product	Target bacteria	Website
Applied Bio Research Inc.	Smart phage	Not specified	http://www.appliedbioresearch.co/
Intralytix Inc.	ListShield™	*Listeria monocytogenes*	http://www.intralytix.com/
	EcoShield™	*Escherichia coli* O157:H7	
	SalmoFresh™[a]	*Salmonella*	
Micreos Food Safety	Listex™ 100[a]	*L. monocytogenes*	http://www.micreos.com/
	SALMONELEX™	*Salmonella*	
Omnilytix	AgriPhage™	*Xanthomonascampestrispv. vesicatoria, or Pseudomonas syringaepv. tomato.*	http://www.omnilytics.com/

[a]FDA (Food and Drug Administration, USA) approved as GRAS (Generally Recognised as Safe).

incidence of phage resistance (McIntyre, Hudson, Billington, & Withers, 2007). Phages may have limited stability under different environmental conditions, and their ability to withstand adverse conditions differs substantially from one phage to another. Therefore, it is important to ensure phage stability and infectivity throughout the storage period of the product to which they are added (Gill & Hyman, 2010). Furthermore, selection of phage that can be propagated using a non-pathogenic or surrogate host is ideal from a safety standpoint when large quantities of phage are required for commercial application in food (Hagens & Loessner, 2010).

It is crucial that the full genome sequence of the phage is known to guarantee the absence of genes encoding virulence factors, which may ultimately lead to more virulent pathogenic strains by insertion of these genes in the genome of host cells (Hagens & Loessner, 2010; Mahony, Mcauliffe, Ross, & Van Sinderen, 2011). Likewise, phage should also have a low frequency of general transduction, the process by which host genes are inserted alongside phage-genetic material in the phage capsid during replication (Ikeda & Tomizawa, 1965). By avoiding phage with these tendencies, the likelihood of transferring virulence factors from one host to another is negligible.

Most of the current phage applications for biocontrol described in the literature and recommended for commercial phage products involve spraying or direct addition to food surfaces. Dilution and development of phage resistance are major problems that may arise following direct application of phages to food (Hagens & Loessner, 2010). It has been suggested that the addition of large numbers of phages and regular, effective sanitation of equipment can help overcome these two problems. It is agreed that spraying phages in sufficiently high numbers could theoretically remove all the target bacterial cells from the food surface; however, this may be costly and allow the development of niches where phage counts are low and phage resistance may develop. Thus, it has been proposed that immobilisation of phages on food packaging materials may offer an interesting, alternative approach for application of phages (Anany, Chen, Pelton, & Griffiths, 2011).

Immobilisation of phages for detection and therapeutic applications has been discussed in many research publications and patent applications (Table 4.2). For instance, phage-containing capsules or tablets for therapeutic applications in human and veterinary medicine could be produced by immobilisation of phages on a solid matrix, such as skim milk or whey protein, before embedding them in a solid support such as carbohydrate or cellulose-based materials (Murthy & Engelhardt, 2012). It was also claimed that this approach could be used to incorporate phage in animal feed to reduce the shedding of pathogens. In another potential approach for immobilisation, T7, T4 and λ phages were encapsulated and spun in polymer nanofibers (Salalha, Kuhn, Dror, & Zussman, 2006). The phages were released after the polymer fibers were dissolved and retained their infectivity. A similar study was done using coaxial electrospinning where T4 phage was pre-encapsulated in alginate before being electrospun into fibers (Korehei & Kadla, 2013). The phage maintained its full activity over a long storage time. A broad host range, lytic *Salmonella* Enteritidis phage (Φ-PVP-SE1) retained infectivity following encapsulation in a water-in-oil-in-water (W/O/W) multiple emulsion (Balcão et al., 2009). Recently, different phages were encapsulated in alginate and gelatine capsules as an approach to overcome the low stomach pH upon oral

Table 4.2 Different approaches for phage immobilisation

Immobilisation approach	Phage	Notes	References
Physical immobilisation	Filamentous phages (e.g. IG40, Lm P4:A8); *Salmonella* and *Staph. aureus* phages; T4	Used in biosensors for detection purposes	Balasubramanian, Sorokulova, Vodyanoy and Simonian (2007); Bennett, Davids, Vlahodimou, Banks, and Betts (1997); Lakshmanan et al. (2007); Nanduri, Balasubramanian, Sista, Vodyanoy, and Simonian (2007); Nanduri, Bhunia, Tu, Paoli, and Brewster (2007); Nanduri, Sorokulova et al. (2007)
	Phages[a]	• Immobilisation of freeze-dried phage on solid cellulose-based material • Phage is stabilised by skim milk and whey protein	Murthy and Engelhardt (2008)
	Staph. aureus; *Pseudomonas aureoginosa* phages	• Immobilisation on biodegradable polyester microsphere • Used as dry powder to control lung diseases	Puapermpoonsiri, Spencer, and Van Der Walle (2009)
Chemical immobilisation	*Salmonella* phages	Chemically biotinylated phage immobilised onto streptavidin-labeled magnetic beads for detection purpose	Sun, Brovko, and Griffiths (2001)
	T4	• Immobilisation on gold by hydrogen bonding	Singh et al. (2009)

Continued

Table 4.2 Continued

Immobilisation approach	Phage	Notes	References
		• Used in biosensors for detection purposes	
	Siphoviridae phages (*Staph. aureus*)	Immobilisation on microcrystals using glutamine or glycine	Alvarez-Gonzalez et al. (2012)
Encapsulation/ Electrospinning	*Staph. aureus* phages K	Co-encapsulation with calcium carbonate in alginate beads	Yongsheng et al. (2012)
	Escherichia coli O157:H7 phages	Encapsulation in methacrylate polymer	Stanford et al. (2010)
	Felix O1 (*Salmonella* phages)	Microencapsulation in chitosan-alginate microspheres	Ma et al. (2008)
	Salmonella phage cocktail	Microencapsulation in sodium alginate	Jiayi et al. (2010)
	Phage CA933P (STEC and EHEC)	Encapsulation in alginate and pectin	Dini, Islan, de Urraza, and Castro (2012)
	Phages[a]	Encapsulation of immobilisation. Freeze-dried phages on skim milk with stearic and palmitic acids	Murthy and Engelhardt (2008)
	T7, T4	Electrospinning	Salalha et al. (2006)
	T4	Electrospinning (phages were encapsulated in alginate before spinning)	Korehei and Kadla (2013)

Site-specific immobilisation	T4	Immobilisation of phages onto streptavidin-labeled magnetic beads (using genetically modified phage)	Tolba et al. (2010)
	Phages[a]	Immobilisation of phages on modified surfaces (nylon, cellulose, etc.). Using coupling agents to create covalent bonds between phages heads and substrate	Hugh and Michael (2009)
	Phages[a]	Immobilisation of phages on modified surfaces with free amine terminal moiety	Evoy et al. (2009)
	E. coli and *Listeria monocytogenes* phages	Immobilisation of phages on modified cellulose membranes (based on charge difference)	Anany et al. (2011)
	E. coli, *Shigella*, *Salmonella* and *L. monocytogenes* phages	Immobilisation of phages on modified silica beads (based on charge difference)	Cademartiri et al. (2010)

[a]Unspecified phage name or type.

ingestion to promote their use as therapeutic agents (Jiayi et al., 2010; Ma et al., 2008; Stanford et al., 2010; Ma et al., 2012; Zhang et al., 2010).

In all the aforementioned protocols, the phages are immobilised inside a protective material and their antimicrobial activity is realised upon the release of phages from these materials. This format is more suitable for therapeutic application of phages (e.g. providing protection of phages against the acidic pH of the stomach following their oral administration) but its use in foods is limited to situations in which release of encapsulated phages can be triggered by a specific event (e.g. a change in temperature). These failings have prompted interest in site-specific oriented immobilisation of phages on solid supports through their heads to leave tail fibers free to recognise receptors on the host cell. The use of immobilised phages may also alleviate concerns over the addition of 'viruses' to food, as immobilisation of phages on packaging materials will ensure that they will only be released into the food when the target pathogen is present and poses a threat to public health. In this context, our group has developed an easy method for phage immobilisation in the desired orientation on modified cellulose membranes based on charge differences between heads and tail components of phages. These phage-based, bioactive membranes carrying cocktails of *E. coli* O157:H7 or *L. monocytogenes* phages were able to effectively control the growth of their target bacteria in raw and ready-to-eat meats, respectively, under different storage temperatures and atmospheric conditions (Anany et al., 2011; Cademartiri et al., 2010). In more recent research, our group has shown that immobilised *L. monocytogenes*-specific phage cocktail and the commercially available *L. monocytogenes* phage preparation, LISTEX P100 were able to significantly reduce the count of *L. monocytogenes* in cantaloupe and RTE meat (unpublished results). Other protocols can be used to achieve the site-specific immobilisation of phages using genetic (Tolba, Minikh, Brovko, Evoy, & Griffiths, 2010) or chemical modification of head proteins or the substrate surface (Evoy, Singh, & Glass, 2009; Hugh & Michael, 2009).

4.5 The use of phage endolysins as biocontrol agents in food

The idea of using bacteriophages as biocontrol agents to combat bacterial pathogens has been rekindled after the increased prevalence of antibiotic-resistant strains, which has become a major concern for public health. However, the use of the whole phage is facing some challenges due to development of resistance against phage infection, host range limitations and in some cases phages may play a role in bacterial transduction of heterologous virulence factors (Bergan, DyveLingelem, Simm, Skotland, & Sandvig, 2012; Callewaert, Walmagh, Michiels, & Lavigne, 2011).

Endolysins, on the other hand, are enzymes encoded by the phage genome and synthesised in phage-infected cells at the end of the reproduction cycle to break down peptidoglycan bonds. The result of this activity is degradation of the rigid murein layer and release of progeny phages (Loessner, 2005). Most tailed phages lyse the host cell by the production of two lysis proteins: endolysin and holin. Holins are small

hydrophobic proteins encoded by the phage and function to form membrane lesions or holes through which the endolysins can then pass, which causes an instant lysis (Wang, Smith, & Young, 2000).

Many researchers have investigated the use of these enzymes to control bacterial pathogens. Since they attack the peptidoglycan backbone, it is important to note that endolysins can also act exogenously in Gram-positive bacteria since the peptidoglycan is, in most cases, accessible from without. In Gram-negative cells where the presence of the outer membrane prevents access by hydrophilic lytic enzymes, it becomes difficult for endolysins to work effectively (Loessner, 2005). However, when the lipopolysaccharide layer is disrupted, cells become sensitive to external murein hydrolases (Callewaert et al., 2011; Fischetti, 2010). The mode of action for endolysins is purely enzymatic; thus, no development of bacterial resistance has been reported so far. Due to their specificity, chemical nature and activity, there are various applications for endolysins that could be utilised.

One of the obvious applications of endolysins is the biocontrol of pathogens and spoilage bacteria in food and feed by directly adding the purified enzyme to the food or to the raw product (Loessner, 2005; Schmelcher, Donovan, & Loessner, 2012). Genetic modification of fermentative bacteria such as *Lactococcus* spp. to produce active endolysin to kill contaminating bacteria has been investigated (Turner, Waldherr, Loessner, & Giffard, 2007). However, in this case the target spoilage bacteria must be sensitive to the secreted lysin. Another interesting application for phage endolysins is to achieve resistance to phytopathogenic bacteria through the development of transgenic plants that express endolysin genes. For example, potato plants expressing T4 endolysin are resistant to *Erwinia carotovora* (de Vries et al., 1999). Upon destruction of cells by *Erwinia* pectinases, the T4 lysozyme is released from plant tissues and hydrolyses the bacterial cells. However, an effect on other bacteria was observed when the endolysin was released from roots (Ahrenholtz, Harms, de Vries, & Wackernagel, 2000).

Endolysins may also be used as therapeutic agents, either alone or in combination with classical antibiotics. Studies revealed the potential to control infection by streptococci (Cheng, Nelson, Zhu, & Fischetti, 2005; Loeffler, Nelson, & Fischetti, 2001; Nelson, Loomis, & Fischetti, 2001) and *Bacillus anthracis* (Loessner, 2005). Other endolysins, such as PlyV12 from an *Enterococcus faecalis* phage (Yoong, Schuch, Nelson, & Fischetti, 2004) and Ply3626 from a *Clostridium perfringens* phage, may have use for the treatment of some mucosal and other infections in animals and humans (Zimmer, Vukov, Scherer, & Loessner, 2002).

Endolysins in general are composed of two domains: the hydrolytic domain, which is responsible for the enzymatic activity of the lysin, and the cell-wall binding domain (CBD), which is responsible for attachment to the peptidoglycan layer and thus responsible for host specificity. The high affinity of CBDs can be harnessed to immobilise bacterial pathogens and remove them from a certain environment (food). If combined with fluorescent labels such as GFP (green fluorescent protein), it could be also used for detection purposes (Callewaert et al., 2011; Fischetti, 2010; Schmelcher et al., 2012). Nevertheless, some challenges related to stability, specificity and purification are hindering the widespread use of endolysins.

4.6 Combining bacteriophages with other preservation techniques to enhance food safety

The encouraging results obtained from studies on the use of phages for biocontrol, coupled with the increasing number of commercially available phage-based products, does not mean that phages are the magic bullet that could work solely to control foodborne pathogens. Thus, combinations of phage treatment and other currently available food safety intervention strategies to produce a synergistic killing effect need further investigation.

The ability of phages to decontaminate stainless steel and polypropylene surfaces inoculated with *L. monocytogenes* was investigated (Roy, Ackermann, Pandian, Picard, & Goulet, 1993). It was found that phage treatment alone was able to achieve approximately a 3.0 $\log_{10}$ cycle drop in cell number, while a combination of phage and 40 ppm of a quaternary ammonium compound (QUATAL) resulted in a 5.0 $\log_{10}$ unit reduction in the attached bacterial cells. Similarly, application of phages in combination with nisin was investigated to control *L. monocytogenes* in fresh-cut honeydew melon (Leverentz et al., 2003). Separate treatment of samples with phages and nisin resulted in 4.6 and 3.2 $\log_{10}$ unit reductions in count, respectively, when compared with untreated samples. However, when the two treatments were combined, a 5.7 $\log_{10}$ unit reduction in count of the target pathogen was observed. Atterbury et.al. demonstrated that a greater reduction of *Campylobacter* numbers on chicken skin was achieved when phages (φ2) were applied in combination with freezing (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003), which is known to reduce the number of *Campylobacter* in processed chicken (Sampers, Habib, De Zutter, Dumoulin, & Uyttendaele, 2010).

Recently, a combination of *E. coli* lytic phage cocktail (BEC8) and the essential oil *trans*-cinnameldehyde (TC) was investigated to control growth of three *E. coli* O157:H7 strains in whole romaine lettuce and baby spinach leaves at inoculum levels of 4.0, 5.0 or 6.0 $\log_{10}$ CFU/leaf during storage at 4, 8, 23 or 37 °C (Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011). Application of TC and phage separately caused a reduction in *E. coli* viable cells by 3.0 and 1.0 $\log_{10}$ CFU/leaf, respectively, at 8 °C after 24 h storage. When essential oil and phage were applied together at the same incubation temperature, the cell numbers were below those detectable by the assay after 10 min and 1 h at all inoculation levels for spinach and lettuce, respectively. The authors observed changes in the long-chain unsaturated fatty acids in the cell membranes of *E. coli* in the presence of TC, which in turn could facilitate phage attachment and transfer of the nucleic acid of the phage into the host. As a result, the phage hijacked the host cell much faster than for cells with unaltered membranes.

In another study, a cocktail of six *Salmonella*-specific phages was used in combination with an antagonistic bacterium, *Enterobacter asburiae* JX1, to control multiple *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saintpaul and Typhimurium CDT104) on sprouting mung beans and alfalfa seeds incubated for 4 days at 25 °C (Ye, Kostrzynska, Dunfield, & Warriner, 2010). There were around 3.0 and 5.0 $\log_{10}$ unit reductions in *Salmonella*

count in mung beans when compared to the untreated controls after phage and *E. asburiae* JX1 treatments, respectively. On the other hand, when the phage and the bacterium were applied together, *Salmonella* counts were reduced to below the detection limit of the method used for counting in this study. Similar results were achieved with the sprouting alfalfa seeds. In a similar study, *Listeria* phages were used in combination with a proactive culture (*Lactobacillus sakei* TH1) to reduce *L. monocytogenes* on sliced, cooked ham (Holck & Berg, 2009). Phages alone caused a ten-fold reduction of *L. monocytogenes* in the tested samples, while using phages and proactive culture together resulted in a 100-fold reduction in count after 14−28 days of storage. Our group also found that adding a *L. monocytogenes*-specific lytic phage cocktail to vacuum-packed RTE oven-roasted turkey breast samples caused more than a 4.0 $\log_{10}$ CFU/gm reduction in *L. monocytogenes* counts after 15 days storage at 4 °C when compared with the untreated samples. On the other hand, aerobically packed, phage-treated samples showed only about a 1.5 $\log_{10}$ unit reduction in *Listeria* counts after incubation for a similar period and temperature (Anany et al., 2011). In addition, our group has recently investigated the use of bacteriophages and high hydrostatic pressure (HHP) to inactivate *Shigella flexneri* in ground beef and *Vibrio cholerae* in salmon and mussels (unpublished results). Inoculated food samples were treated with HPP from 150 to 550 MPa for various times, with phages (cocktail of either three *S. flexneri* or single *V. cholera* phages at 10^9 PFU/ml) or combinations of both HHP and phages. Using HPP at 550 MPa for 5 min, or a more energy-efficient treatment of 350 MPa for 5 min, followed by addition of phages resulted in complete eradication of *S. flexneri* and *V. cholerae* in minced beef and seafood, respectively. The counts of the organisms were reduced to below detectable levels, and growth of the organism was not observed following overnight enrichment of the HPP/phage-treated samples in tryptic soy broth (TSB) (unpublished results).

To support the aforementioned idea of the recommended use of phages in combination with other approaches to control the growth of food-borne bacterial pathogens, it was found that a combination of phages and antibiotic treatments resulted in better recovery of patients with staphylococcal sepsis, septic infection of lungs and osteomyelitis than when they are used alone (Kutateladze & Adamia, 2010).

References

Ackermann, H. W. (2007). 5500 Phages examined in the electron microscope. *Archives of Virology*, *152*, 227−243.

Ackermann, H. W., & Prangishvili, D. (2012). Prokaryote viruses studied by electron microscopy. *Archives of Virology*, *157*, 1843−1849.

Ahrenholtz, I., Harms, K., de Vries, J., & Wackernagel, W. (2000). Increased killing of *Bacillus subtilis* on the hair roots of transgenic T4 lysozyme-producing potatoes. *Applied and Environmental Microbiology*, *66*, 1862−1865.

Alvarez-Gonzalez, E., Alfadhel, M., Mane, P., Ford, S. J., Moore, B. D., & Van Der Walle, C. F. (2012). Bioprocessing of bacteriophages via rapid drying onto microcrystals. *Biotechnology Progress*, *28*, 540−548.

Anany, H., Chen, W., Pelton, R., & Griffiths, M. W. (2011). Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat by using phages immobilized on modified cellulose membranes. *Applied and Environmental Microbiology*, *77*, 6379–6387.

Anderson, T. F. (1992). Electron microscopry of phages. In J. Cairns, G. S. Stent, & J. D. Watson (Eds.), *Phage and origins of molecular biology* (pp. 63–78). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products. *Microbial Biotechnology*, *2*, 601–612.

Atterbury, R. J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., & Connerton, I. F. (2003). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Applied and Environmental Microbiology*, *69*, 6302–6306.

Balasubramanian, S., Sorokulova, I. B., Vodyanoy, V. J., & Simonian, A. L. (2007). Lytic phage as a specific and selective probe for detection of *Staphylococcus aureus* – a surface plasmon resonance spectroscopic study. *Biosensors and Bioelectronics*, *22*, 948–955.

Balcão, V. M., Azeredo, J., Castro, L. M., Costa, C. I., Santos, S., Matos, C. M., et al. (November 2009). A multiple emulsion formulation of bacteriophage encapsulated in lipid nanovesicles. Book of Abstract of MicroBiotec 2009 *Vilamoura*, 28–30.

Bennett, A. R., Davids, F. G. C., Vlahodimou, S., Banks, J. G., & Betts, R. P. (1997). The use of bacteriophage-based systems for the separation and concentration of *Salmonella*. *Journal of Applied Microbiology*, *83*, 259–265.

Bergan, J., Dyve Lingelem, A. B., Simm, R., Skotland, T., & Sandvig, K. (2012). Shiga toxins. *Toxicon*, *60*, 1085–1107.

Brovko, L. Y., Anany, H., & Griffiths, M. W. (2012). Bacteriophages for detection and control of bacterial pathogens in food and food-processing environment. In J. Henry (Ed.), *Advances in Food and Nutrition Research*. *67*, 241–288.

Cademartiri, R., Anany, H., Gross, I., Bhayani, R., Griffiths, M., & Brook, M. A. (2010). Immobilization of bacteriophages on modified silica particles. *Biomaterials*, *31*, 1904–1910.

Callewaert, L., Walmagh, M., Michiels, C. W., & Lavigne, R. (2011). Food applications of bacterial cell wall hydrolases. *Current Opinion in Biotechnology*, *22*, 164–171.

Cheng, Q., Nelson, D., Zhu, S., & Fischetti, V. A. (2005). Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme. *Antimicrobial Agents and Chemotherapy*, *49*, 111–117.

Coffey, B., Mills, S., Coffey, A., Mcauliffe, O., & Ross, R. P. (2010). Phage and their lysins as biocontrol agents for food safety applications. *Annual Review of Food Science and Technology*, *1*, 449–468.

Dini, C., Islan, G. A., de Urraza, P. J., & Castro, G. R. (2012). Novel biopolymer matrices for microencapsulation of phages: enhanced protection against acidity and protease activity. *Macromolecular Bioscience*, *12*, 1200–1208.

Duckworth, D. H. (1976). Who discovered bacteriophage. *Bacteriological Reviews*, *40*, 793–802.

Evoy, S., Singh, A., & Glass, N. R. (2009). *Bacteriophage immobilization for biosensors*. US Patent 12/466,089.

Fischetti, V. A. (2010). Bacteriophage endolysins: a novel anti-infective to control gram-positive pathogens. *International Journal of Medical Microbiology*, *300*, 357–362.

Fruciano, D. E., & Bourne, S. (2007). Phage as an antimicrobial agent: d'Herelle's heretical theories and their role in the decline of phage prophylaxis in the West. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *18*, 19–26.

Galvez, A., Abriouel, H., Benomar, N., & Lucas, R. (2010). Microbial antagonists to food-borne pathogens and biocontrol. *Current Opinion in Biotechnology*, *21*, 142–148.

Garcia, P., Rodriguez, L., Rodriguez, A., & Martinez, B. (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science and Technology*, *21*, 373−382.

Gill, J. J., & Hyman, P. (2010). Phage choice, isolation, and preparation for phage therapy. *Current Pharmaceutical Biotechnology*, *11*(1), 2−14.

Goodridge, L. D., & Bisha, B. (2011). Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage*, *1*, 130−137.

Greer, G. G. (2005). Bacteriophage control of foodborne bacteria. *Journal of Food Protection*, *68*(5), 1102−1111.

Guttman, B., Raya, R., & Kutter, E. (2005). Basic phage biology. In E. Kutter, & A. Sulakvelidze (Eds.), *Bacteriophages: Biology and applications* (pp. 29−66). Boca Raton, Florida: CRC Press.

Hagens, S., & Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Applied Microbiology and Biotechnology*, *76*, 513−519.

Hagens, S., & Loessner, M. J. (2010). Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Current Pharmaceutical Biotechnology*, *11*, 58−68.

Himmelweit, F. (1945). Combined action of penicillin and bacteriophage on staphylococci. *Lancet*, *249*, 104−105.

Holck, A., & Berg, J. (2009). Inhibition of *Listeria monocytogenes* in cooked ham by virulent bacteriophages and protective cultures. *Applied and Environmental Microbiology*, *75*, 6944−6946.

Hugh, S., & Michael, M. (2009). *Immobilisation and stabilisation of virus*. US Patent 10/512,635.

Ikeda, H., & Tomizawa, J.-I. (1965). Transducing fragments in generalized transduction by phage P1: I. Molecular origin of the fragments. *Journal of Molecular Biology*, *14*, 85−109.

Jiayi, Z., Kraft, B. L., Yanying, P., Wall, S. K., Saez, A. C., & Ebner, P. D. (2010). Development of an anti-*Salmonella* phage cocktail with increased host range. *Foodborne Pathogens and Disease*, *7*, 1415−1419.

Jończyk, E., Klak, M., Miedzybrodzki, R., & Górski, A. (2011). The influence of external factors on bacteriophages-review. *Folia Microbiologica*, *56*, 191−200.

Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel natural food antimicrobials. In M. P. Doyle, & T. R. Klaenhammer (Eds.), *Annual review of food science and technology* (Vol. 3). Palo Alto: Annual Reviews.

Korehei, R., & Kadla, J. (2013). Incorporation of T4 bacteriophage in electrospun fibres. *Journal of Applied Microbiology*, *114*, 1425−1434.

Kutateladze, M., & Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, *28*, 591−595.

Kutter, E., & Sulakvelidze, A. (2005). *Bacteriophages − Biology and applications − Introduction*. Boca Raton: Crc Press-Taylor & Francis Group.

Lakshmanan, R. S., Guntupalli, R., Hu, J., Kim, D.-J., Petrenko, V. A., Barbaree, J. M., et al. (2007). Phage immobilized magnetoelastic sensor for the detection of *Salmonella typhimurium*. *Journal of Microbiological Methods*, *71*, 55−60.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., et al. (2003). Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*, 4519−4526.

Loeffler, J. M., Nelson, D., & Fischetti, V. A. (2001). Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. *Science*, *294*, 2170−2172.

Loessner, M. J. (2005). Bacteriophage endolysins − current state of research and applications. *Current Opinion in Microbiology*, *8*, 480−487.

Mahony, J., Mcauliffe, O., Ross, R. P., & Van Sinderen, D. (2011). Bacteriophages as biocontrol agents of food pathogens. *Current Opinion in Biotechnology*, *22*, 157−163.

Ma, Y., Pacan, J. C., Wang, Q., Xu, Y., Huang, X., Korenevsky, A., et al. (2008). Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. *Applied and Environmental Microbiology*, *74*, 4799−4805.

Ma, Y., Pacan, J. C., Wang, Q., Sabour, P. M., Huang, X., & Xu, Y. (2012). Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing *Staphylococcus aureus* intestinal carriage. *Food Hydrocolloids*, *26*, 434−440.

McIntyre, L., Hudson, J. A., Billington, C., & Withers, H. (2007). Biocontrol of bacteria: past, present and future strategies. *Food New Zealand*, *7*, 25−32.

McIntyre, L., Hudson, J. A., Billington, C., & Withers, H. (2012). Biocontrol of foodborne bacteria. In A. Mcelhatton, & P. J. Do Amaral Sobral (Eds.), *Novel technologies in food science*. New York: Springer.

Murthy, K., & Engelhardt, R. (2008). *Stabilized bacteriophage formulations*. WO/2006/047870.

Murthy, K., & Engelhardt, R. (2012). *Encapsulated bacteriophage formulation*. US Patent 13/528,215.

Nanduri, V., Balasubramanian, S., Sista, S., Vodyanoy, V. J., & Simonian, A. L. (2007). Highly sensitive phage-based biosensor for the detection of β-galactosidase. *Analytica Chimica Acta*, *589*, 166−172.

Nanduri, V., Bhunia, A. K., Tu, S.-I., Paoli, G. C., & Brewster, J. D. (2007). SPR biosensor for the detection of L. monocytogenes using phage-displayed antibody. *Biosensors and Bioelectronics*, *23*, 248−252.

Nanduri, V., Sorokulova, I. B., Samoylov, A. M., Simonian, A. L., Petrenko, V. A., & Vodyanoy, V. (2007). Phage as a molecular recognition element in biosensors immobilized by physical adsorption. *Biosensors and Bioelectronics*, *22*, 986−992.

Nelson, D., Loomis, L., & Fischetti, V. A. (2001). Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proceedings of the National Academy of Sciences*, *98*, 4107−4112.

Nurmemmedov, E., Castelnovo, M., Catalano, C. E., & Evilevitch, A. (2007). Biophysics of viral infectivity: matching genome length with capsid size. *Quarterly Reviews of Biophysics*, *40*, 327−356.

Pennazio, S. (2006). The origin of phage virology. *Rivista Di Biologia*, *99*, 103−129.

Prigent, M., Leroy, M., Confalonieri, F., Dutertre, M., & Dubow, M. S. (2005). A diversity of bacteriophage forms and genomes can be isolated from the surface sands of the Sahara Desert. *Extremophiles*, *9*, 289−296.

Puapermpoonsiri, U., Spencer, J., & Van Der Walle, C. F. (2009). A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. *European Journal of Pharmaceutics and Biopharmaceutics*, *72*, 26−33.

Roy, B., Ackermann, H. W., Pandian, S., Picard, G., & Goulet, J. (1993). Biological inactivation of adhering *Listeria monocytogenes* by listeriaphages and a quaternary ammonium compound. *Applied and Environmental Microbiology*, *59*, 2914−2917.

Salalha, W., Kuhn, J., Dror, Y., & Zussman, E. (2006). Encapsulation of bacteria and viruses in electrospun nanofibres. *Nanotechnology*, *17*, 4675−4681.

Sampers, I., Habib, I., De Zutter, L., Dumoulin, A., & Uyttendaele, M. (2010). Survival of *Campylobacter* spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *International Journal of Food Microbiology*, *137*, 147−153.

Scharff, R. L. (2012). Economic burden from health losses due to foodborne illness in the United States. *Journal of Food Protection*, *75*, 123−131.

Schmelcher, M., Donovan, D. M., & Loessner, M. J. (2012). Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, *7*, 1147−1171.

Sillankorva, S. M., Oliveira, H., & Azeredo, J. (2012). Bacteriophages and their role in food safety. *International Journal of Microbiology*, *2012*, 863945.

Singh, A., Glass, N., Tolba, M., Brovko, L., Griffiths, M., & Evoy, S. (2009). Immobilization of bacteriophages on gold surfaces for the specific capture of pathogens. *Biosensors and Bioelectronics*, *24*, 3645−3651.

Snowdon, A., Buzby, J. C., & Roberts, T. (2002). Epidemiology, cost, and risk of foodborne disease. In D. O. Cliver, & H. P. Riemann (Eds.), *Foodborne diseases* (2nd ed.). New York, NY: Elsevier Press Ltd.

Stanford, K., Mcallister, T. A., Niu, Y. D., Stephens, T. P., Mazzocco, A., Waddell, T. E., et al. (2010). Oral delivery systems for encapsulated bacteriophages targeted at *Escherichia coli* O157:H7 in feedlot cattle. *Journal of Food Protection*, *73*, 1304−1312.

Summers, W. C. (1993). How bacteriophage came to be used by the phage group. *Journal of the History of Biology*, *26*, 255−267.

Sun, W. L., Brovko, L., & Griffiths, M. W. (2001). Use of bioluminescent *Salmonella* for assessing the efficiency of constructed phage-based biosorbent. *Journal of Industrial Microbiology and Biotechnology*, *27*(2), 126−128.

Tolba, M., Minikh, O., Brovko, L. Y., Evoy, S., & Griffiths, M. W. (2010). Oriented immobilization of bacteriophages for biosensor applications. *Applied and Environmental Microbiology*, *76*, 528−535.

Turner, M. S., Waldherr, F., Loessner, M. J., & Giffard, P. M. (2007). Antimicrobial activity of lysostaphin and a *Listeria monocytogenes* bacteriophage endolysin produced and secreted by lactic acid bacteria. *Systematic and Applied Microbiology*, *30*, 58−67.

Vanhelvoort, T. (1992). The controversy between John H. Northrop and Max Delbrück on the formation of bacteriophage: bacterial synthesis or autonomous multiplication? *Annals of Science*, *49*, 545−575.

Viazis, S., Akhtar, M., Feirtag, J., & Diez-Gonzalez, F. (2011). Reduction of *Escherichia coli* O157:H7 viability on hard surfaces by treatment with a bacteriophage mixture. *International Journal of Food Microbiology*, *145*, 37−42.

de Vries, J., Harms, K., Broer, I., Kriete, G., Mahn, A., During, K., et al. (1999). The bacteriolytic activity in transgenic potatoes expressing a chimeric T4 lysozyme gene and the effect of T4 lysozyme on soil- and phytopathogenic bacteria. *Systematic and Applied Microbiology*, *22*(2), 280−286.

Wang, I.-N., Smith, D. L., & Young, R. (2000). HOLINS: the protein clocks of bacteriophage infections. *Annual Review of Microbiology*, *54*, 799−825.

Ye, J., Kostrzynska, M., Dunfield, K., & Warriner, K. (2010). Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *Journal of Food Protection*, *73*, 9−17.

Yoong, P., Schuch, R., Nelson, D., & Fischetti, V. A. (2004). Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Journal of Bacteriology*, *186*, 4808−4812.

Zhang, J., Kraft, B. L., Pan, Y., Wall, S. K., Saez, A. C., & Ebner, P. D. (2010). Development of an anti-*Salmonella* phage cocktail with increased host range. *Foodborne Pathogens and Disease*, *7*, 1415−1419.

Zimmer, M., Vukov, N., Scherer, S., & Loessner, M. J. (2002). The murein hydrolase of the bacteriophage φ3626 dual lysis system is active against all tested clostridium perfringens strains. *Applied and Environmental Microbiology*, *68*, 5311−5317.

Bacteriophages as antimicrobials in food products: applications against particular pathogens

5

H. Anany[1,2], *L.Y. Brovko*[1], *T. El Arabi*[2], *M.W. Griffiths*[1]
[1]University of Guelph, Guelph, ON, Canada; [2]Ain Shams University, Cairo, Egypt

5.1 Introduction

As noted in Chapter 4, renewed interest has been focused on natural antimicrobials as an alternative to 'chemical' preservatives to improve safety, enhance hygienic quality and extend shelf life of foods (Juneja, Dwivedi, & Yan, 2012). Generally natural antimicrobials can be divided into two major categories:

1. Compounds isolated from animals, plants or microorganisms with pronounced antibacterial properties against a wide range of bacterial pathogens and food-spoilage organisms. These include essential oils, bacteriocins and enzymes/proteins such as lysozymes, lactoferrin, protamine and endolysins.
2. Living organisms, including antagonistic bacteria (Galvez, Abriouel, Benomar, & Lucas, 2010) and bacteriophages (Coffey, Mills, Coffey, Mcauliffe, & Ross, 2010; Garcia, Rodriguez, Rodriguez, & Martinez, 2010).

Of these two approaches, the latter may offer an advantage, as evolution has endowed these organisms with the ability to adapt to various environments, allowing them to exert their natural antibacterial properties in complex matrices such as food. Furthermore, both bacteria and bacteriophages are easy to propagate, which reduces the cost of their production.

Bacteriophages (phages) as antimicrobials provide another favourable feature: they target specific bacteria, and hence provide an opportunity to tailor the treatment toward the selected bacterium and/or group of bacteria. This feature may be especially important for production of foods that involve fermentation, as a pathogen-specific phage will not inactivate the starter cultures responsible for the fermentation.

Bacteriophages are abundant in the environment, and we consume them daily in large numbers with food and drink. They are considered as safe, and some bacteriophages are listed by US Food and Drug Administration (FDA) as Generally Recognised As Safe (GRAS), which allows producers to use them as processing aids to control *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat meats or raw red meats (Sillankorva, Oliveira, & Azeredo, 2012).

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Building on Chapter 4, this chapter gives some examples for using bacteriophages to target specific food-borne pathogens:

- Gram-negative pathogens such as *Campylobacter*, *E. coli* and *Salmonella*
- Gram-positive pathogens such as *Clostridium botulinum* and *L. monocytogenes*

Each section reviews the characteristics of the relevant pathogen and current research on the effectiveness of bacteriophages in controlling the pathogen.

5.2 Bacteriophages to control Gram-negative food-borne pathogens

5.2.1 Campylobacter

The *Campylobacter* genus is comprised of Gram-negative, spiral, microaerophilic bacteria and it contains 17 species and 6 recognized subspecies (Connerton, Timms, & Connerton, 2011). Among these species, 8 have been identified as potential human pathogens, with *Campylobacter jejuni* being considered as the major causal agent of *Campylobacter*-associated gastrointestinal illnesses around the world, and in some instances can result in serious complications, such as Guillain-Barré syndrome (Griffiths & Park, 1990; Mandrell et al., 2005).

Due to its colonization of the chicken intestine in relatively high numbers, *Campylobacter* cells can readily contaminate poultry products during processing, and as a result these products are considered to be an important source of *Campylobacter* infection in humans (Berrang, Buhr, & Cason, 2000; Jorgensen et al., 2002; Shane, 2000). Moreover, *Campylobacter* cells can be rapidly spread in broiler house flocks within 2 days of initial exposure due to their ability to colonise the avian gut, even when present at low numbers, and to survive in faeces (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003a; Young, Ziprin, Hume, & Stanker, 1999).

One of the suggested approaches to tackle this food-borne pathogen was the application of phages throughout the poultry-processing chain (Connerton et al., 2011). Phages could be administered to the live chicken as a therapeutic agent or applied directly to the poultry meat and processing surfaces. In this context, two *Campylobacter*-specific lytic phages were administered to young broilers before and after infection with *C. jejuni* to study their prophylactic and therapeutic effects, respectively (Wagenaar, Van Bergen, Mueller, Wassenaar, & Carlton, 2005). Phages administered therapeutically (following infection) resulted in about a 3.0 $\log_{10}$ unit reduction in the *Campylobacter* count in faecal content after 48 h of phage treatment when compared with the untreated control group. On the other hand, phages administered prior to infection (prophylactic treatment) resulted only in a delay in the colonization of the chicken intestine by *Campylobacter*. There was then an initial 2.0 $\log_{10}$ unit reduction before the bacterial behaviour followed a comparable pattern to the therapeutic group by showing only around 1.0 $\log_{10}$ unit difference in the bacterial count within a week. In both cases, the phage application did not produce any side effects in the treated chickens.

In another study, lytic *Campylobacter*-specific phages, CP8 and CP34, were mixed with an antacid, $CaCO_3$, before oral administration at different MOI to 25-day-old broiler chickens, which were artificially colonized with *C. jejuni* (Loc Carrillo et al., 2005). It was observed that the reduction in *Campylobacter* caecal count ranged from 1.5 to 5.0 $\log_{10}$ CFU/g when compared with counts from untreated broilers and the optimum phage dose was 7.0 $\log_{10}$ PFU. Similarly, CP220 phage was found to cause a 2.0 $\log_{10}$ CFU/g reduction in caecal counts of both *C. jejuni* and *Campylobacter coli* 48 h after the chickens received a phage dose of 7.0 and 9.0 $\log_{10}$ PFU, respectively (El-Shibiny et al., 2009). Alternatively, phages could be applied directly to the poultry products as biocontrol agents. For example, the effect of phage φ2 at different concentrations on the recovery of *C. jejuni* NCTC 12,662 from artificially contaminated fresh and frozen chicken skin was investigated (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003b). It was found that high phage concentrations (7.0 $\log_{10}$ PFU) caused a significant reduction in *Campylobacter* recovery at all sampling points in both fresh and frozen samples when compared with untreated samples. Furthermore, the ability of two lytic *Campylobacter*-specific phages, CP8 and CP30, to reduce attachment and biofilm formation by *C. jejuni* on glass at 37 °C under microaerophilic conditions was assessed (Siringan, Connerton, Payne, & Connerton, 2011). After 24 h of phage treatment, a 1.0−3.0 $\log_{10}$ CFU/cm^2 reduction in the viable count of *C. jejuni* was observed compared to the untreated samples.

5.2.2 Cronobacter *spp.*

Cronobacter spp. are Gram-negative and motile pathogenic bacilli belonging to the family Enterobacteriaceae (Chai et al., 2012). They were identified as *Enterobacter sakazakii* (Farmer, Asbury, Hickman, & Brenner, 1980) before Iversen et al. (2007), proposed a reclassification of these bacteria into a new genus called *Cronobacter*. The new genus contained six species (*Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis* and *Cronobacter genomospecies* 1) (Iversen et al., 2007; Zuber et al., 2008). Based on partial 16S ribosomal DNA and *hsp*60 sequencing, another species (*Cronobacter condiment*) was recently added and *C. genomospecies* 1 was renamed *Cronobacter universalis* (Joseph et al., 2011). *Cronobacter* spp. are opportunistic pathogens and consumption of infant formula contaminated with this bacterium can result in necrotizing enterocolitis (NEC), sepsis and meningitis with a mortality rate of 40−80% in infants less than 4 weeks old, with low birth weight, premature babies being particularly vulnerable to infection (Gurtler, Kornacki, & Beuchat, 2005; Mullane et al., 2007). Only *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been associated with reported cases in neonates (Healy et al., 2010). Moreover, due to its opportunistic nature, *Cronobacter* infection has also been reported in the elderly and immunocompromised adults (Gosney, Martin, Wright, & Gallagher, 2006; See, Than, & Tang, 2007).

As powdered infant formula is a non-sterile food product, it is critical to safely handle and store the reconstituted formula and instigate control measures to eliminate this pathogenic bacterium (Zuber et al., 2008). Refrigeration of the reconstituted milk

and consuming it as soon as possible after reconstitution are important recommended control measures for *Cronobacter* spp. On the other hand, different commercial biocide formulations, chemical disinfectants, essential oils, polyphenols and prebiotics have been investigated to control the dissemination of *Cronobacter* strains and prevent biofilm formation (Amalaradjou & Venkitanarayanan, 2011; Kim, Weng, Silva, Jung, & Marshall, 2010; Quintero et al., 2011; Yan et al., 2012). However, the search for new, natural and more effective agents to control this organism, such as phage, may ultimately benefit the industry and consumers. In this context, the ability of two lytic *C. sakazakii* phages (ESP1-3 and ESP 732-1) to control their host in reconstituted infant formula was investigated at different incubation temperatures (Kim, Klumpp, & Loessner, 2007). High concentrations of both phages (around 10^9 PFU/mL) were able to eradicate their corresponding *C. sakazakii* strains from the reconstituted infant formula after incubation at 24 °C, while ESP 732-1 was more efficient than ESP 1−3 at 12 and 37 °C at eradicating their hosts. In a similar study, a cocktail of five lytic phages (F, 23, 81, 82 and 83) was able to control the growth of 35 out of 40 tested *Cronobacter* strains in artificially inoculated infant formula at MOI of 10^6 within 4 h of incubation at 30 °C, while addition of phage at a lower MOI (10^4) did not inhibit growth of the tested strains (Zuber et al., 2008). Abbasifar studied the efficacy of a cocktail of five lytic *Cronobacter* phages to control the pathogen in infant formula (Abbasifar, 2013). A cocktail of these five phages present in equal amounts (10^8 PFU/mL) was added to both tryptone soy broth (TSB) and reconstituted infant formula (RIF) specially prepared for premature neonates and infants inoculated with 10^1−10^4 CFU/mL of three *C. sakazakii* strains and subsequently incubated at 24 °C. In the presence of the phage cocktail, *C. sakazakii* counts remained below detectable levels (<10 CFU/mL) for 24 h following inoculation in both TSB and RIF, whereas counts in the absence of phage were $>10^6$ CFU/mL.

5.2.3 Escherichia coli

Escherichia coli is one of the predominant Gram-negative bacteria in both human and animal gut. Generally, *E. coli* strains are harmless to their host; however, pathogenic strains exist, which cause diseases in humans and animals. These strains have been grouped based on their unique virulence factors. The pathogenic groups have been designated as enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), diffusely adherent (DAEC) and enteroaggregative (EAggEC) (Bhunia, 2008; Hartland & Leong, 2013; Kaper, Nataro, & Mobley, 2004; Willshaw, Cheasty, & Smith, 2000). It was reported that, even among healthy individuals, the fatality rate of EHEC infection compared to other *E. coli* infections is high (Hagens & Loessner, 2010). *E. coli* O157:H7 is an EHEC strain that has resulted in many sporadic and outbreak-associated hemorrhagic colitis cases around the world (Reiss, Kunz, Koin, & Keeffe, 2006) and is of concern because of its high morbidity and mortality rates. The infectious dose of *E. coli* O157:H7 is as few as 10 cells and in severe infection, damage may occur to the kidneys resulting in the potentially fatal haemolytic uremic syndrome (HUS), which is most commonly observed in young children (O'Flynn, Ross, Fitzgerald, & Coffey, 2004). Although

different control methods have been introduced to control pathogenic *E. coli*, outbreaks are still pervasive (Scallan et al., 2011) and are associated with many foods such as undercooked beef, raw milk, as well as cold sandwiches, water, unpasteurised apple juice, sprouts and leafy green vegetables (Feng, 1995; Guh et al., 2010; Rasko et al., 2011). Cattle and other ruminants are the main reservoir for *E. coli* O157:H7 (Callaway, Carr, Edrington, Anderson, & Nisbet, 2009). In 2011, a Shiga-toxin producing *E. coli* strain caused a large outbreak in Germany involving more than 3800 reported cases (Loos et al., 2012). The associated strain was serotype O104:H4 and was found to produce Shiga-toxin 2 as well harbouring virulence traits of enteroaggregative *E. coli* (EAggEC) (Bielaszewska et al., 2011; Rohde et al., 2011). Other serotypes of Shiga-toxin producing *E. coli* (STEC), specifically O26, O111, O103, O121, O45 and O145, have recently been added to the list of adulterants in meat by USDA-FSIS and have raised issues regarding their detection (Brooks et al., 2005; Debroy, Roberts, Valadez, Dudley, & Cutter, 2011).

Published studies have described the use of phage cocktails as biocontrol agents for pathogenic *E. coli*. In a study by O'Flynn et al. (2004) a phage cocktail containing three *E. coli* O157:H7-specific phages (e11/2, e4/1c and pp01) was used to control *E. coli* O157:H7 in raw beef samples at MOI of around 10^6 (O'Flynn et al., 2004). The bacterial cells were eradicated from the surface of seven out of nine beef samples after 1 h incubation at 37 °C. When the challenge experiment was done in broth medium, a 5.0 $\log_{10}$ cycle reduction in the cell number was noticed after 3 h of incubation at the same temperature. These results indicate that food matrices have an effect on the activity of the applied phage (Rees & Dodd, 2006). In another study, different concentrations of a cocktail of three *Myoviridae* phages (ECP-100) lytic for *E. coli* O157:H7 significantly reduced the number of viable host cells on artificially inoculated hard surfaces (glass coverslips and gypsum boards) and food (tomato, spinach, broccoli and ground beef) (Abuladze et al., 2008). The observed reduction in count ranged from 85% to 100% on hard surfaces, while on foods the values ranged from 94% to 100%. The same cocktail caused a significant reduction of *E. coli* O157:H7 in contaminated fresh-cut iceberg lettuce and cantaloupe after incubation at 4 °C for 2 and 7 days, respectively (Sharma, Patel, Conway, Ferguson, & Sulakvelidze, 2009). In a similar study, a lytic phage cocktail, BEC8, was investigated to control the growth and attachment of *E. coli* O157:H7 to different food contact surfaces, such as stainless steel, ceramic tile and high-density polyethylene (Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011). Phage cocktail was added at 10^6 PFU per chip, which was inoculated with bacterial concentrations ranging from 10^6 to 10^4 CFU and incubated at 10, 12, 23 and 37 °C for 10 min, 1 h and 24 h. Counts of the organism were below the detection limit (10 CFU/chip) of the assay used for enumeration on all tested surfaces following phage application at MOI of 100 and storage at 23 and 37 °C for 1 h and 10 min, respectively. Phages can also be used to control the pathogen on agricultural equipment. The population of *E. coli* O157:H7 was reduced by 4.5 $\log_{10}$ cycle on spinach harvester blades after application of a cocktail of specific lytic phages (Patel, Sharma, Millner, Calaway, & Singh, 2011). Application of three *E. coli* phage cocktails, EcoM-AG2, EcoM-AG3 and EcoM-AG10, to contaminated raw meat resulted in a

reduction of *E. coli* O157:H7 below the assay detection limit (1.0 $\log_{10}$ CFU/mL) when the samples were stored aerobically at 4 °C for 12 days (Anany, Chen, Pelton, & Griffiths, 2011). However, when the meat samples were incubated at 10 °C, significant reductions in the recovered *E. coli* cells from the contaminated samples have only been noticed after 7 and 9 days with around 1.0 and 1.5 $\log_{10}$ unit reduction, respectively, when compared with the control. Recently, a cocktail of 140 specific lytic *E. coli* phages, active against more than 430 EHEC and non-EHEC strains, was used to control growth and biofilm formation by pathogenic *E. coli* in different food products (lettuce, cabbage, ground meat and egg) when applied by spraying or dipping (Jassim, Abdulamir, & Abu Bakar, 2012). This study showed that complete elimination of EHEC strains was achieved within 48 h of incubation at 4 °C from the contaminated food samples, while more than a 3.0 $\log_{10}$ CFU/mL reduction in counts of cells present in biofilm was observed on glass slides and catheters. In addition, it was found that spraying 10 contaminated eggs or 200 g of the contaminated vegetables or ground meat samples with a phage cocktail containing 10^5 PFU/mL produced a similar reduction in counts of *E. coli* as that obtained by immersing the same samples in 400 mL of the phage cocktail (approximately 10^3 PFU/mL) for 20 s. Moreover, validation of the efficacy of a commercial anti-*E. coli* O157:H7 phage product, Ecoshield, was performed using lettuce and beef contaminated with around 2×10^3 CFU/g *E. coli* O157:H7 (Carter et al., 2012). The phage preparation was applied using a spray gun adjusted to achieve a final phage concentration of around 10^6 PFU/g in meat and 10^5 and 10^6 PFU/g in lettuce. Ecoshield, which contains three different lytic phages, caused a reduction of 94−98% viable cells in ground beef after storage at refrigeration temperature for 7 days post-grinding. However, the initial addition of phages did not protect from recontamination after grinding, which was suggested to be due to reduced accessibility of phages to bacteria. The bacterial load in lettuce was reduced by 87% under similar storage conditions when a high concentration of phages was initially applied.

5.2.4 Salmonella

Salmonella has been considered to be one of the most important etiological agents of food-borne illness around the world. Since its discovery in 1885, *Salmonella* has been associated with many outbreaks of food-borne illness in different countries (Bell & Kyriakides, 2002). *Salmonella* spp. are Gram-negative, facultative anaerobic and usually motile small rods. The identification of surface antigens and phage typing has led to the current recognition of almost 2400 serotypes under two species of *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. Most food-borne serotypes belong to the species *S. enterica* (D'Aoust, 2001). *Salmonella* strains are often part of the natural flora in chicken and turkey. Therefore, during slaughter of colonized animals, meat is regularly contaminated. In addition, eggs may carry the bacterium, either due to contamination of the ovaries and oviduct of layers or by deposition of faeces on the eggshell (Moffatt & Musto, 2013).

Salmonellosis results from the ingestion of *Salmonella*-contaminated food products such as poultry and dairy products. The symptoms range from mild to severe

gastroenteritis to enteric fever. In more severe cases, the organism can migrate into the blood stream and/or the lymphatic system and result in bacteraemia or septicaemia, which may result in serious disorders such as osteomyelitis, cardiac inflammation and/or neural disorders (D'Aoust, 1994). The development of multiple-antimicrobial resistant strains of *Salmonella* has increased the severity of the problem. For instance, *Salmonella* Typhimurium DT104 in Europe and North America was reported to be resistant to at least five antibiotics, enhancing its role in many recent outbreaks of severe gastrointestinal infections (Besser et al., 2000). Furthermore, novel resistant serotypes with no recognised phage type have emerged and reported to be associated with food-borne illness (Davis et al., 2007). *Salmonella* Typhimurium DT104 outbreaks have been associated with various food items, such as unpasteurized dairy products, pork sausage, chicken, meat paste and fresh apple cider. It represented about 38% of the human reported cases of salmonellosis in 2000 in Canada (Doré et al., 2004). One well-known incident of salmonellosis occurred in 2006, when 31 people became ill due to *S.* Montevideo contamination of Cadbury's chocolate, resulting in a recall of approximately one million chocolate bars that cost the company over £20 million (Carroll, 2009). In one of the worst food-borne outbreaks in the United States, 529 people became infected and 8 died due to contamination of peanut products with *Salmonella* Typhimurium, which led to an estimated $1000 million loss in peanut sales (Cavallaro et al., 2011; Medus et al., 2009). In 2010, consumption of contaminated eggs resulted in a large *S. enteritidis* outbreak, with more than 1800 reported cases and recall of around 500,000,000 eggs (CDC, 2010).

In addition to utilizing food safety management programs during production and processing, formulation of the food products to create growth-limiting conditions can be applied to control *Salmonella* and prevent its adverse effects (Bell & Kyriakides, 2002). Promising results have been obtained when phages were used to control growth of *Salmonella* in food (Coffey et al., 2010; Hagens & Loessner, 2010; Rees & Loessner, 2005). In one example, cheddar cheese was made from raw and pasteurised milk artificially inoculated at a level of 10^4 CFU/mL of milk with a *Salmonella enteritidis* strain that had been associated with an outbreak linked to a snack product (Lunchmates) containing cheese (Ahmed et al., 2000). *Salmonella* phage SJ2 (10^8 CFU/mL) was added to the milk prior to cheese manufacture and cheese was ripened for a period of 99 days at 8 °C (Modi, Hirvi, Hill, & Griffiths, 2001). In cheeses made from milk containing phages, *Salmonella* did not survive in the pasteurised milk cheeses beyond 89 days of ripening, whereas counts of about 50 CFU *Salmonella*/g were observed in raw milk cheeses, even after ripening for 99 days. However, in contrast, counts of *Salmonella* in all the cheeses made from milk not containing phages were 10^3 CFU/g after 99 days. In another study, three lytic *Salmonella* phages significantly reduced the number of *Salmonella enteritidis* PT4 recovered from contaminated chicken skin stored for 9 days at 5 °C (Fiorentin, Vieira, & Barioni Júnior, 2005). In similar studies, application of a high phage dose as a surface decontaminant for chicken skins reduced the recovery of *Salmonella* from the treated skin by 2.0−5.6 $\log_{10}$ units (Goode, Allen, & Barrow, 2003; Kang, Kim, Jung, & Woo, 2013; Lopez-Cuevas et al., 2012). The efficacy of a phage cocktail (PCI) to control *Salmonella* Typhimurium U288 on pig

skin was also investigated (Hooton, Atterbury, & Connerton, 2011). After storage for 4 days at 4 °C, a significant reduction in *Salmonella* count (>99%) was observed for the phage-treated samples. In addition, a cocktail of four lytic *Salmonella* phages was able to decrease numbers of *Salmonella enteritidis* by 3.5 $\log_{10}$ CFU/g on artificially contaminated honeydew melon slices stored at 5 or 10 °C and by approximately 2.5 $\log_{10}$ CFU/g on slices stored at 20 °C (Leverentz et al., 2001). The same study showed that the cocktail did not effectively control the growth of *Salmonella* on apple slices, possibly due to the instability of phages at the acidic pH of apple.

A broad host range *Salmonella* phage, Felix O1, and a related phage variant were used to control *Salmonella* Typhimurium DT104 on chicken frankfurters (Whichard, Namalwar, & Pierson, 2003). It was revealed that under certain environmental conditions a reduction of *Salmonella* cells by 1.8−2.1 $\log_{10}$ units could be achieved. Other studies involving ready-to-eat (RTE) meats showed that when phages infecting *Salmonella* Typhimurium PT160 were added at different titres to various densities of host bacteria inoculated onto raw and cooked beef, there was a significant inactivation of the pathogen both at 5 °C (2−3 $\log_{10}$ CFU/g reduction in count) and at 24 °C (>5.9 $\log_{10}$ CFU/g reduction in count) (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008). Different foods (turkey deli meat, chocolate milk, hot dog, seafood and egg yolk) contaminated with *S.* Typhimurium were treated with a single lytic phage, FO1-E2, at a MOI of around 10^5 and incubated at 8 and 15 °C for 6 days (Guenther, Herzig, Fieseler, Klumpp, & Loessner, 2012). The levels of *Salmonella* were below the detection limit in all treated food samples stored at 8 °C, while the bacterial count was reduced by 5.0 $\log_{10}$ units on turkey deli meat and in chocolate milk and by 3 $\log_{10}$ units on hotdog and in seafood at 15 °C.

The use of two broad host range *Salmonella* phages (SSP5 and SSP6) to control *Salmonella* Oranienburg on experimentally contaminated alfalfa seeds was unsuccessful, perhaps due to the low MOI applied (Kocharunchitt, Ross, & Mcneil, 2009). This study emphasized the importance of the concentration of the applied phage, as using a high phage titre will increase the chance of the phage meeting the target bacterium to initiate the infection. In another biocontrol study for environmental application of phages, a cocktail of three lytic phages (sww65, sww275 and sww297) at different concentrations was evaluated for its potential to control growth of *Salmonella* strains at 30 and 37 °C in wastewater (Turki, Ouzari, Mehri, Ammar, & Hassen, 2012). The addition of the phage cocktail (MOI of 10^4) resulted in a significant reduction in growth of all the tested *Salmonella* strains at both temperatures, as evidenced by an inability to detect the *Salmonella* virulence gene *invA* by PCR. Interestingly, this study showed also that phage application generally reduced the number of Enterobacteriaceae in the treated wastewater samples.

5.2.5 Shigella

Shigella is another important food-borne pathogen belonging to the Enterobacteriaceae family. It is a Gram-negative, non-motile facultative anaerobic rod. According to

genetic studies, it is more related to *Escherichia* than *Salmonella* (Jay, 2000). Biochemical and serological characteristics can be used to differentiate four main species under the genus *Shigella*: *Shigella sonnei*, *Shigella flexneri*, *Shigella boydii* and *Shigella dysenteriae*. Although all the species have the ability to cause human disease, only the first three are generally regarded as food-borne pathogens. Humans are the main reservoir of infection and the infectious dose may be as low as 10 CFU (Kothary & Babu, 2001; Sutherland & Varnam, 2002). Shigellosis is a food-borne disease that is developed within 12−48 h after the ingestion of *Shigella*-contaminated food, whereupon it colonizes and penetrates the colonic epithelial cells. The initial symptoms include fever, aches, fatigue and loss of appetite, which may be associated with watery diarrhoea that may manifest into bloody stools or dysentery. In certain severe cases, fatal HUS may develop due to the production of Shiga-toxin (Acheson, 2001).

Although shigellosis is more common in tropical, developing countries where hygiene standards are low, it is also reported in developed countries. It has been estimated that *Shigella* causes around 131,254 illnesses, 1456 hospitalization and 10 deaths each year in the United States (Scallan et al., 2011). The problem has increased due to the high prevalence of antimicrobial resistant strains among the *Shigella* strains isolated in the United States (Sivapalasingam et al., 2006). Many foods have the potential to be contaminated, so a wide range of food products has been implicated as vehicles of shigellosis transmission. Generally, where hygiene standards are poor, foods that receive significant handling during preparation, such as salads, soft cheese, vegetables and meat products, are at the greatest risk of contamination by *Shigella* (Acheson, 2001). In this context, a *S. sonnei* outbreak was reported among air travellers who departed from Hawaii on 12 separate flights to Japan, Australia, 22 US States and American Samoa (Gaynor et al., 2009). It was estimated that between 300 and 1500 passengers were infected and raw carrot served onboard was considered as the likely vehicle of infection. This outbreak illustrates the risk of rapid, global spread of illness from a single point of contamination. In order to control this pathogen, several approaches can be taken, such as minimize hands-on procedures, exclude potential excretors of *Shigella* from handling food, and ensure good standards of personal hygiene (Sutherland & Varnam, 2002).

Most of the reported work with *Shigella* phages has evaluated their use as prophylactic or therapeutic agents alone or in combination with antibiotics (Kutateladze & Adamia, 2008, 2010; Kutter et al., 2010; Merril, Scholl, & Adhya, 2003; Sulakvelidze & Barrow, 2005; Sulakvelidze, Alavidze, & Morris, 2001). Interestingly, *Shigella* phages active against *S. flexneri* and *S. sonnei* have been formulated in tablet form, which is now commercially available for treatment and prophylaxis of dysentery in all age groups as well as high-risk groups (http://www.biochimpharm.ge/). As *Shigella* is commonly spread through faecal−oral transmission and this spread may be through a contaminated food product, the use of phages on these food materials or to clean contaminated surfaces in food processing plants has been proposed to control spread of this pathogen. However, only one recent study investigated the application of *Shigella* phages at different concentrations to control the growth of pathogenic *Shigella* spp. (*S. flexneri 2a*, *S. dysenteriae*, *S. sonnei*) in contaminated RTE, spiced chicken stored at 4 °C for 72 h (Zhang, Wang, & Bao, 2013). When phages were

applied at a high MOI (around 10^5), up to a 2.0 $\log_{10}$ CFU/g reduction in count was observed after 24 h, and the population of the target pathogen was below the detection limit of the assay used for enumeration by the end of the storage period.

5.2.6 Vibrio

Vibrio spp. are Gram-negative, mesophilic bacilli and are among the microbiota of marine fish species; during warm weather they increase in number in filter-feeding shellfish living in coastal waters (Lacroix, 2011). *Vibrio*-associated outbreaks are more predominant among populations who consume insufficiently cooked or raw seafood products. For instance, raw and lightly marinated fish contaminated with *V. cholera* O1 caused a large outbreak in Peru, which spread throughout most of Latin America and resulted in around 450,000 illnesses and 4000 deaths (Fields, Popovic, Wachsmuth, & Olsvik, 1992). It was reported that around 50% of the 2500 reported cases of illness in the United States from 1984 to 1993 was due to the consumption of bivalve molluscs contaminated with *Vibrio* spp. (mainly *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholera*) (Wittman & Flick, 1995). In a similar study in Australia, it was found that *V. parahaemolyticus* and *V. cholera* were associated with outbreaks linked to cooked prawns, while *V. vulnificus* was found in oyster-related outbreaks during the period from 1990 to 2000 (Sumner & Ross, 2002). *V. parahaemolyticus* and *V. cholera* cause acute diarrhoea in humans, while *V. vulnificus* is associated with gastroenteritis and primary septicaemia (Dziejman & Yildiz, 2011; Gulig, Bourdage, & Starks, 2005).

Although phages can be considered a promising tool to enhance the safety of seafood before consumption, more attention has been focused on the application of *Vibrio* phages as therapeutic agents to treat local and systemic diseases in humans and filter-feeding shellfish, such as shrimps and oysters. In this context, phages were used as a therapeutic agent against *V. vulnificus* in an animal model (Cerveny, Depaola, Duckworth, & Gulig, 2002). Intravenous injection of a high-phage dose, 10^8 PFU/mL, caused optimum protection against systemic and local infection caused by the target pathogen. The study also showed that one phage requires seawater to be able to infect its host, so application of this phage to control *Vibrio* spp. in filter-feeding shellfish such as oysters is not out of the question. The ability of siphoviridae *Vibrio harveyi*-specific lytic phages to protect shrimp larvae (*Penaeumonodon*) from luminous vibriosis was investigated under laboratory and hatchery conditions (Vinod et al., 2006). In the laboratory trial, a phage suspension (around 10^9 PFU/mL) was added to 18-day-old shrimps that were artificially inoculated with *V. harveyi*. It was found that 80% of shrimp larvae survived after two doses of phage treatment as compared with a survival rate of only 25% in untreated samples. While in the hatchery trial, where phage was added daily at a rate of 2×10^5 PFU/mL in water, the survival rate was 86% in treated shrimp larvae and 17% for the untreated ones. Interestingly, treatment with antibiotics resulted in only 40% survival. In a similar hatchery trial, four lytic phages against *V. harveyi* were added to infected shrimp larvae at a concentration of 2×10^6 PFU/mL and the survival rate was compared to antibiotic (oxytetracycline and kanamycin) treatment (Karunasagar, Shivu, Girisha, & Krohne, 2007).

The phage application resulted in 85% survival of larvae, while the antibiotic treatment resulted in a survival rate from 65% to 67%. However, other promising lytic phages of different morphology have been isolated from different sources, characterized and were able to control growth of *Vibrio* spp. *in vitro* (Alagappan, Deivasigamani, Somasundaram, & Kumaran, 2010; Crothers-Stomps, Høj, Bourne, Hall, & Owens, 2010; Phumkhachorn & Rattanachaikunsopon, 2010; Shivu, Rajeeva, Girisha, Krohne, & Karunasagar, 2007; Srinivasan, Ramasamy, Brennan, & Hanna, 2007).

5.3 Bacteriophages to control Gram-positive food-borne pathogens

5.3.1 Bacillus cereus

Bacillus cereus is a Gram-positive, catalase-positive, spore-forming bacterium and a very important etiological agent of food-borne illness. It has a very high infectious dose, 10^5 organisms per gram of food. Hence, the disease usually occurs when a food is highly contaminated with the bacterium and then consumed (Bartoszewicz, Hansen, & Swiecicka, 2008; Stenfors Arnesen et al., 2008). Spores of *B. cereus* have been found in many foods such as cereals, pulses, vegetables, spices and pasteurised, raw and powdered milk.

Two types of food-borne gastroenteritis are associated with *B. cereus* infection: emetic and diarrhoeal. The emetic illness occurs from *B. cereus* that produces a very stable toxin that is resistant to high temperatures and pH changes. The toxin is pre-formed in food before consumption. The other type is mainly associated with diarrhoea and is mediated by a less stable enterotoxin complex. Diarrhoeal illness is the most common syndrome associated with milk (Ehling-Schulz, Fricker, & Scherer, 2004; Stenfors Arnesen et al., 2008; Tourasse, Helgason, Økstad, Hegna, & Kolstø, 2006). It occurs if large numbers of *B. cereus* cells are consumed and enzymes in the stomach break down the bacterial cells, releasing the toxin, which then will cause illness. The virulence factor of the diarrhoeal syndrome is a hemolytic enterotoxin complex designated hemolysin BL (HBL), nonhemolysin enterotoxin (NHE) and cytotoxin K. It causes hemolysis, dermonecrosis and vascular permeability (Jay, Loessner, & Golden, 2005).

Bacillus cereus is ubiquitously distributed in the environment, including in soil, dust, water, air and decaying matter. Some evidence indicates that this bacterium is even present on the bodies of animals (Hornstra et al., 2007; Priest, Barker, Baillie, Holmes, & Maiden, 2004; Tourasse et al., 2006; Zhou, Liu, He, Yuan, & Yuan, 2008).

The optimum temperature for *B. cereus* growth is 30−37 °C, and also it can grow at temperatures as high as 55 °C and as low as 4 °C. This microorganism is a facultative aerobe; however, in the absence of oxygen, the production of toxin is reduced. It cannot grow below pH 4.5 or above pH 9.3. The spores are resistant to heat (inactivated at 100 °C for 10 min) and other adverse conditions, which enables the organism to survive for prolonged periods in many environments (Andersen Borge et al., 2001; Van Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004).

The *B. cereus* group includes *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. thuringensis*. *Bacillus anthracis*, the causative agent of anthrax, may result in a fatal illness, whereas other species are implicated in food-spoilage as well as food-borne illnesses. However, the greatest concern in the food industry is the psychrotrophic species such as *B. cereus* and *B. weihenstephanensis*, because of their ability to grow in chilled environments (Jay et al., 2005).

Bacteriophages have been suggested as a way to control *B. cereus*. In a study to investigate the efficacy of lytic phages to inhibit the subsequent outgrowth of *B. anthracis* ΔSterne spores, a lytic phage cocktail was sprayed on spores at a ratio of 10^4-10^5 (PFU to CFU). It was found that the phage cocktail was able to inhibit the growth of *B. anthracis* (Walter, 2003). It was also noted in the same study that if the phage was applied at a lower ratio, the effect was diminished and spores were able to germinate. Two *Myoviridae* phages, namely FWLBc1 and FWLBc2, were isolated against *B. cereus* from soil. These phages were able to reduce *B. cereus* levels in mashed potatoes by $>6.0\ \log_{10}$ CFU/mL within 24 h at room temperature, when applied at a high concentration (Lee, Billington, Hudson, & Heinemann, 2011).

Some recent studies also described the isolation and characterization of lytic phages against *B. cereus*, Bcp78 (Lee, Shin, Son, & Ryu, 2012), Bcp1(Bandara, Jo, Ryu, & Kim, 2012) and Bc431v3 (El-Arabi et al., 2013). El-Arabi investigated the efficacy of phage Bc431v3 to control *B. cereus* in powdered and pasteurised milk (El-Arabi, 2011). Results showed that phage Bc431v3 was able to prevent the outgrowth of *B. cereus* spores present in milk powder following reconstitution. The effect was especially pronounced when the reconstituted milk was stored at 8 °C. The phage was slightly more effective when it was present in the water used to reconstitute than when it was added to the milk powder prior to reconstitution. The reduced infectivity of the phage present in the powder was presumably the result of desiccation. However, when the milk was reconstituted immediately after the drying process, the phage was unaffected by dehydration but storage of the powder containing the phage for 3 weeks greatly reduced phage numbers. Nevertheless, some phage particles were able to survive and were able to rapidly infect and multiply in the bacteria and their inhibitory effect was restored. Unfortunately, the phage had little or no effect when the reconstituted milk was stored at room temperature. El-Arabi (2011) also inoculated pasteurised milk (skim milk, 2% and 3.25%) with *B. cereus* or *B. weihenstephanensis* spores and the milks were subsequently stored at 8 °C or 6 °C, respectively. Addition of phage Bc431v3, at levels similar to or slightly higher than the numbers of *Bacillus* cells added, resulted in the inhibition of outgrowth of the spores of both bacteria, but the effect on *B. weihenstephanensis* spores was more pronounced. The fat content of the milk did not have any effect on the efficiency of the phage infection.

The ability of phage to control *Bacillus* spp. is limited by the ability of the bacteria to form spores. However, El-Arabi (2011) showed that phage particles could adsorb onto bacterial spores possibly through hydrophobic interactions, particularly with the exosporium layer of the spore, and prevent the outgrowth of the spores even in the presence of nutrients in the environment.

Other factors such as host range limitations and presence of toxins in phage preparations also impede the widespread use of phage to control *B. cereus*. Therefore,

many researchers have focused on the use of phage products, such as endolysins, to combat bacteria instead of using the whole phage. The feasibility of using endolysins to control bacteria is dependent on the proper identification and successful cloning of endolysin genes.

Endolysin proteins from *B. cereus*phages Bastille, TP21 and 12826 were found to be restricted to the peptidoglycan types of the genus *Bacillus*. However, the highest activity of these endolysins was recorded against the *B. cereus* group. The growth of *B. cereus* cells was inhibited in the presence of these endolysins, which confirmed their potential for use in food and medical applications (Loessner, Maier, Daubek-Puza, Wendlinger, & Scherer, 1997). When a group of 10 mice was injected intraperitoneally (IP) with 10^7 *B. cereus* cells, which were highly related to *B. anthracis*, 9 animals died within 4 h (10% survival). However, when mice were treated after 15 min of bacterial inoculation with a 100 μg dose of endolysin PlyG (isolated from phage γ), only 5 animals out of 18 died (73% survival). It was also noted that mortality of 3 of the dead animals was delayed by >24 h (Fischetti, 2005). Another endolysin, PlyPH, was found to be incredibly stable at pH between 4.5 and 10, and was highly specific against *B. anthracis* ΔSterne and *B. cereus* strain RSVF1. The maximum activity of the PlyPH endolysin was achieved at pH between 4.5 and 8, suggesting that PlyPH could be used as an alternative form of therapy (Yoong, Schuch, Nelson, & Fischetti, 2006).

5.3.2 Clostridium botulinum, Clostridium difficile *and* Clostridium perfringens

Clostridium spp. are Gram-positive, anaerobic, spore-forming bacilli. Some species of this genus are involved in food-borne illness and these include *C. perfringens*, *C. difficile* and *C. botulinum*. The illness caused by *Clostridium* spp. ranges from mild, such as the gastroenteritis caused by *C. perfringens*, to fatal as in botulism caused by *C. botulinum*.

Clostridium perfringens is a natural inhabitant of the gastrointestinal tract of many animals. Thus, its occurrence in food is usually through faecal contamination caused by inadequate hygiene of the food handler (Silva et al., 2013). Gastroenteritis produced by *C. perfringens* is mediated by a toxin produced in the stomach following ingestion of a large number of bacterial cells contaminating the food. Symptoms are usually mild and the illness is self-limiting. There are two types of illness caused by *C. perfringens*: type A, the most common type, known to cause typical symptoms of food-borne illness in humans; and type C, known to produce a rare but often fatal illness known as Pig-bel or necrotic enteritis, which is associated with eating temperature-abused cooked meats (Silva et al., 2013; Wahl, Rømma, & Granum, 2013).

Botulism, on the other hand, is associated with the ingestion of a pre-formed neurotoxin in food that is contaminated by *C. botulinum* (Ting & Freiman, 2004). Food-borne botulism is rare but often fatal. The botulinum neurotoxin is heat labile; therefore, the illness usually occurs because of inadequate cooking of food. Foods that generally have minimum oxygen content (anaerobic) such as canned foods,

rich meat soups, thick steak and puddings are the most common food vehicles (Ting & Freiman, 2004). Botulism is clinically manifested by symmetrical cranial nerve palsies that may be followed by paralysis of voluntary muscles, which later progresses to respiratory failure and death (Benecke, 2012; Fujinaga, Sugawara, & Matsumura, 2013).

Clostridium difficile is a common cause of nosocomial diarrhoea (Bartlett, Onderdonk, Cisneros, & Kasper, 1977). *Clostridium difficile* inhabits the human gastrointestinal tract and results in 'antibiotic-related diarrhoea' (Jawa & Mercer, 2012; Songer, 2004). Due to the frequent use of antibiotics in hospitals and other care facilities, the natural bowel flora is disturbed and allows *C. difficile* to predominate and grow to large numbers, resulting in the production of toxins. However, *C. difficile* has been isolated from food animals, especially dairy calves, but it is also found in piglets. The organism has also been isolated from foods, leading to the suggestion that the rise in community-acquired infections is linked to food-borne transmission (Gould & Limbago, 2010) as well as increased virulence (Jawa & Mercer, 2012; Songer, 2004).

Some investigators have sought to challenge these organisms with their specific bacteriophages to control them. However, it seems that because of the special conditions needed to grow these organisms in laboratories as well as their ability to form endospores, only very few publications have reported the use of phages to control *Clostridium* spp.

In a study to control necrotic enteritis (NE) caused by *C. perfringens* in broiler chickens, Miller and his collaborators developed a lytic bacteriophage cocktail, named INT- 401, and used it to prevent NE in *C. perfringens*-challenged broiler chickens (Miller, Skinner, Sulakvelidze, Mathis, & Hofacre, 2010). The phage cocktail was delivered to chickens by two means, through feed or by oral gavage. Both means of administering the phage treatment resulted in a reduced mortality rate in challenged chickens; and even in comparison with toxoid-type vaccine, it seemed to be significantly better. In another trial, several lytic phages belonging to families *Siphoviridae* and *Podoviridae* were isolated against *C. perfringens*, and their endolysins were tested against the bacterium. Results showed that the characterized endolysins were able to hydrolyse cell walls of *C. perfringens* and could be used as highly potent biocontrol agents for this pathogen (Seal, 2013).

In regards to biocontrol of *C. difficile*, in the last 15 years only two major studies were reported. The endolysin of phage Ø CD27 (namely Ø CD27L) was tested for the ability to hydrolyse the cell walls of *C. difficile*. Interestingly, the enzyme was able to eliminate bacterial cells in buffers and in milk (Mayer, Narbad, & Gasson, 2008). In a batch-fermentation study, *C. difficile* was challenged with phage Ø CD27. Even though it is a lysogenic phage, nevertheless, it was able to significantly reduce and even prevent toxin production by *C. difficile* (Meader et al., 2010).

Numerous studies have reported the use of phage-display libraries to generate peptides and antibodies to neutralize botulinum toxin (Amersdorfer & Marks, 2000; Yu et al., 2009; Zdanovsky & Zdanovskaia, 2013). However, no reported studies

were found in the PubMed database on application of phages or their endolysins to control cells and spores of *C. botulinum*.

5.3.3 L. monocytogenes

The genus *Listeria* currently consists of 10 species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi* (Rocourt & Buchrieser, 2007), *L. marthii* (Graves et al., 2010), *L. rocourtia* (Leclercq et al., 2010), *L. weihenstephanensis* (Lang Halter, Neuhaus, & Scherer, 2013) and *L. fleischmannii* (Bertsch et al., 2013). They are Gram-positive, facultative anaerobic, non-spore forming and motile rod-shaped bacteria. Although there are a few reports that *L. ivanovii* and *L. seeligeri* infect humans, *L. monocytogenes* is regarded as the major cause of human illness. It is an opportunistic pathogen that is able to colonize the intestine and cause listeriosis (Donnelly, 2001). Listeriosis usually causes flu-like symptoms including fever, muscle aches and gastrointestinal symptoms such as nausea or diarrhoea while in high-risk groups, including pregnant women, neonates, elderly and immunocompromised persons, the disease may lead to fatal disorders such as meningitis, bacteraemia and abortion in pregnant women (Swaminathan & Gerner-Smidt, 2007). Although listeriosis is a rare disease when compared with other food-borne illnesses, it has a high mortality rate (20−30%), especially in high-risk groups (Todd & Notermans, 2011). The Centers for Disease Control and Prevention (CDC) reported that there are about 1591 confirmed cases of severe listeriosis each year in the United States; of these about 255 die (Scallan et al., 2011). *Listeria monocytogenes* contains 14 serotypes, but 1/2a, 1/2b and 4b are the most common serotypes associated with food-borne illness (Donnelly, 2001). It is considered to be an important food-borne pathogen as it has the ability to survive at low temperatures, where it can contaminate refrigerated food and RTE foods and can even become endemic in cold storage facilities (Todd & Notermans, 2011). In this context, several large outbreaks of listeriosis have been related to contaminated fresh and RTE food products, including dairy products, meat, egg products, vegetables and seafood (Farber & Peterkin, 1991). The contamination occurs at different stages during food production and processing and can involve ingredients, factory workers, contaminated processing equipment or the factory environment. For instance, slicers, conveyor belts and sandwich ingredients were found to be among the major sources for *L. monocytogenes* contamination in one Swiss sandwich-producing plant. Moreover, it was found that certain strains persisted for more than nine months on slicers and conveyor belts, which increased the chance of sandwich contamination (Blatter, Giezendanner, Stephan, & Zweifel, 2010).

Several studies have described the biocontrol of *Listeria* in foods using *Listeria* phages. For instance, a broad host range lytic *Listeria* phage, P100, was investigated to control the growth of *L. monocytogenes* in soft cheese during manufacturing (Carlton, Noordman, Biswas, DeMeester, & Loessner, 2005). Bioinformatic analysis of the total genome sequence of P100 did not reveal any similarity to any genes or proteins believed to play a role in the pathogenicity or virulence of *L. monocytogenes*. High doses of P100 reduced the bacterial count to below levels of detection for the

entire ripening time and regrowth was not observed. In another recent study, P100 and A511 *Listeria*-specific phages were used to control *L. monocytogenes* in liquid and solid RTE foods (Guenther, Huwyler, Richard, & Loessner, 2009). It was reported that in liquid foods, such as chocolate milk, bacterial counts rapidly dropped below the level of direct detection after storage at 6 °C for 6 days. While on solid foods (hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage and lettuce leaves), phages could reduce bacterial counts by up to 5.0 $\log_{10}$ units under the same conditions. In addition, application of higher doses of phages (3×10^8 PFU/g) was more effective than lower doses. In another report, the influence of *Listeria* phage P100 dose, contact time and storage temperature on the listericidal activity of the phage on the surface of fresh channel catfish fillet was studied (Soni, Nannapaneni, & Hagens, 2010). It was found that a phage contact time of 30 min reduced *L. monocytogenes* populations by more than 1.0 $\log_{10}$ CFU/g, whereas 15 min contact time with phage yielded less than 1.0 $\log_{10}$ CFU/g reduction in *L. monocytogenes* loads on catfish fillet. An overall reduction in count was detected in the treated samples over a 10 day shelf life at 4 or 10 °C. On the other hand, another study reported that addition of *Listeria* phage LH7 had no significant effect on the presence of two strains of *L. monocytogenes* on artificially inoculated beef stored for 4 weeks at 4 °C (Dykes & Moorhead, 2002). The reason for this negative effect might be that an insufficient phage concentration per unit surface area was applied (it was around 0.5 to 1.0 phage/cm^2) (Hagens & Loessner, 2010). The nature of the food matrix is another factor for low activity of phages in this particular experiment, as the phage might have absorbed to the beef surface and was not able to 'find' its host cell. When a related food product with a smoother surface (i.e. vacuum-packed chicken breast roll) was artificially contaminated with *L. monocytogenes* prior to application of a myoviridae *Listeria* phage at MOI of around 100 and stored at 30 or 5 °C for 1or 21 days, the *L. monocytogenes* count was reduced immediately after the addition of phage by around 1.5 and 2.5 $\log_{10}$ CFU/cm^2 at the two tested temperatures, respectively (Bigot et al., 2011). Although regrowth of the bacterium was observed during storage at 30 °C, the phenomenon did not occur at 5 °C. In another similar study, application of a *Listeria*-specific PCI to artificially contaminated RTE oven roasted turkey breast samples stored under different packaging conditions resulted in a reduction of the *L. monocytogenes* count below the assay detection limit when the meats were stored at 4 °C for 9 days (Anany et al., 2011). However, a 2.0 $\log_{10}$ unit reduction in counts of *L. monocytogenes* was noticed after 9 days storage under vacuum packaging conditions at 25 °C. In a recent study, the efficacy of a commercially available anti-*Listeria* phage preparation, LISTEX P100, was validated when applied alone or in combination with the chemical antimicrobials potassium lactate and sodium diacetate to control *L. monocytogenes* on RTE roast beef and cooked turkey (Chibeu et al., 2013). Application of LISTEX P100 alone resulted in a reduction of *L. monocytogenes* viable cells by about 2.0 $\log_{10}$ CFU/$cm^2$when the meats were stored for 28 days at 4 °C. The combined action of the phage and the chemical antimicrobial reduced the counts of *L. monocytogenes* by more than 2.1 $\log_{10}$ units during storage of both RTE meat products.

Listeria monocytogenes-specific lytic phages have been successfully used in many studies as decontaminating agents to reduce biofilm formation and remove *Listeria*

cells from different food contact surfaces, such as stainless steel and polypropylene, and reductions in count of 3 to more than 5 $\log_{10}$ units were observed in these studies (Hibma, Jassim, & Griffiths, 1997; Montañez-Izquierdo, Salas-Vázquez, & Rodríguez-Jerez, 2012; Roy, Ackermann, Pandian, Picard, & Goulet, 1993).

5.4 Conclusion and future trends

Since the appearance of multi-drug resistant strains of pathogens, there has been a resurgence of interest in the use of bacteriophages as therapeutic and control agents. This has resulted in the appearance of several reviews and at least one book on the topic in the last 6 years, as well as the formation of companies dedicated to producing phages and phage products (Atterbury, 2009; Brovko, Anany, & Griffiths, 2012; Chan, Abedon, & Loc-Carrillo, 2013; Coffey et al., 2010; Garcia, Martinez, Obeso, & Rodriguez, 2008; Hagens & Loessner, 2007, 2010; Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012; Hertwig, Hammerl, Appel, & Alter, 2013; Monk, Rees, Barrow, Hagens, & Harper, 2010; O'Flaherty, Ross, & Coffey, 2009; Oliveira, Azeredo, Lavigne, & Kluskens, 2012; Oliveira, Castilho, Cunha, & Pereira, 2012; Sabour & Griffiths, 2010; Schmelcher, Donovan, & Loessner, 2012; Seckin & Baladura, 2010; Sharma & Goodridge, 2013; Sulakvelidze, 2013).

Despite this increased research activity, the uptake of phage technology for the control of food-borne pathogens by the food industry remains low. The reasons for this may include the lack of bacteriophages with the required spectrum of activity and stability, the possibility of development of resistance to the phage, the opportunity for transduction of virulence genes into the host bacterium, and last but not least consumer perception regarding the addition of 'viruses' to foods. All of these can be overcome. The issue of specificity can be addressed by the use of phage cocktails, and there is the possibility of producing 'designer' phages. Tail fibres and receptor-binding proteins of phages can be cloned and may offer the opportunity to produce ambivalent phages capable of infecting a variety of bacterial hosts (Scholl, Rogers, Adhya, & Merril, 2001). By applying appropriate screening techniques, it is also possible to identify phages that are resistant to, for example, desiccation. The use of phage cocktails will also reduce opportunities for the bacterial host to develop resistance, and by genome sequencing the absence of genes related to lysogeny and bacterial virulence can be confirmed, which will eliminate the threat of transfer of virulence genes to the host. Lastly, although we ingest probably millions of phages each day without any ill effects, consumer fears may be allayed by the adoption of phage immobilization techniques to create packaging materials that ensure that phages are only transferred to the food if the target organism is present. Arguably, the most effective use of phages will be as adjuncts to other processing techniques to provide an extra layer of protection to our food supply.

As we understand more and more about phage biology and as the number of phages that are isolated increases, then opportunities for their use to control and detect pathogens and to combat infections will increase.

References

Abuladze, T., Li, M., Menetrez, M. Y., Dean, T., Senecal, A., & Sulakvelidze, A. (2008). Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, *74*(20), 6230−6238.

Abbasifar, R. (2013). *Biocontrol of Cronobacter spp. using bacteriophage in infant formula* (Ph.D.). University of Guelph.

Acheson, D. W. K. (2001). Shigella. In R.,G. Labbé, & S. Garcia (Eds.), *Guide to foodborne pathogens*. New York, NY: John Wiley and Sons.

Ahmed, R., Soule, G., Demczuk, W. H., Clark, C., Khakhria, R., Ratnam, S., et al. (2000). Epidemiologic typing of *Salmonella enterica* serotype Enteritidis in a Canada-wide outbreak of gastroenteritis due to contaminated cheese. *Journal of Clinical Microbiology*, *38*, 2403−2406.

Alagappan, K. M., Deivasigamani, B., Somasundaram, S. T., & Kumaran, S. (2010). Occurrence of *Vibrio parahaemolyticus* and its specific phages from shrimp ponds in East coast of India. *Current Microbiology*, *61*, 235−240.

Amalaradjou, M. A. R., & Venkitanarayanan, K. (2011). Proteomic analysis of the mode of antibacterial action of trans-cinnamaldehyde against *Cronobacter sakazakii* 415. *Foodborne Pathogens and Disease*, *8*, 1095−1102.

Amersdorfer, P., & Marks, J. D. (2000). Phage libraries for generation of anti-botulinum scFv antibodies. In O. Holst (Ed.), *Bacterial toxins: Methods and protocols*. Humana Press.

Anany, H., Chen, W., Pelton, R., & Griffiths, M. W. (2011). Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat by using phages immobilized on modified cellulose membranes. *Applied and Environmental Microbiology*, *77*, 6379−6387.

Andersen Borge, G. I., Skeie, M., Sørhaug, T., Langsrud, T., & Granum, P. E. (2001). Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. *International Journal of Food Microbiology*, *69*(3), 237−246.

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products. *Microbial Biotechnology*, *2*, 601−612.

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E., & Connerton, I. F. (2003a). Isolation and characterization of *Campylobacter* bacteriophages from retail poultry. *Applied and Environmental Microbiology*, *69*, 4511−4518.

Atterbury, R. J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., & Connerton, I. F. (2003b). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Applied and Environmental Microbiology*, *69*, 6302−6306.

Bandara, N., Jo, J., Ryu, S., & Kim, K.-P. (2012). Bacteriophages BCP1-1 and BCP8-2 require divalent cations for efficient control of *Bacillus cereus* in fermented foods. *Food Microbiology*, *31*, 9−16.

Bartlett, J. G., Onderdonk, A. B., Cisneros, R. L., & Kasper, D. L. (1977). Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in Hamsters. *Journal of Infectious Diseases*, *136*, 701−705.

Bartoszewicz, M., Hansen, B. M., & Swiecicka, I. (2008). The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiology*, *25*, 588−596.

Bell, C., & Kyriakides, A. (2002). Salmonella*: A practical approach to the organism and its control in foods*. Oxford: Blackwell Science.

Benecke, R. (2012). Clinical relevance of botulinum toxin immunogenicity. *BioDrugs*, *26*, e1−e9.

Berrang, M. E., Buhr, R. J., & Cason, J. A. (2000). *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poultry Science*, *79*(2), 286−290.

Bertsch, D., Rau, J., Eugster, M. R., Haug, M. C., Lawson, P. A., Lacroix, C., et al. (2013). *Listeria fleischmannii* sp. nov., isolated from cheese. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 526−532.

Besser, T. E., Goldoft, M., Pritchett, L. C., Khakhria, R., Hancock, D. D., Rice, D. H., et al. (2000). Multiresistant *Salmonella* Typhimurium DT104 infections of humans and domestic animals in the Pacific Northwest of the United States. *Epidemiology and Infection*, *124*(2), 193−200.

Bhunia, A. K. (2008). *Foodborne microbial pathogens: Mechanisms and pathogenesis*. Springer.

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., et al. (2011). Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *The Lancet Infectious Diseases*, *11*, 671−676.

Bigot, B., Lee, W. J., Mcintyre, L., Wilson, T., Hudson, J. A., Billington, C., et al. (2011). Control of *Listeria monocytogenes* growth in a ready-to-eat poultry product using a bacteriophage. *Food Microbiology*, *28*, 1448−1452.

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., & Heinemann, J. A. (2008). Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiology*, *25*(2), 400−406.

Blatter, S., Giezendanner, N., Stephan, R., & Zweifel, C. (2010). Phenotypic and molecular typing of *Listeria monocytogenes* isolated from the processing environment and products of a sandwich-producing plant. *Food Control*, *21*(11), 1519−1523.

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M., et al. (2005). Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983−2002. *Journal of Infectious Disease*, *192*, 1422−1429.

Brovko, L. Y., Anany, H., & Griffiths, M. W. (2012). Bacteriophages for detection and control of bacterial pathogens in food and food-processing environment. In H. Jeyakumar (Ed.), *Advances in food and nutrition research*. Academic Press.

Callaway, T. R., Carr, M. A., Edrington, T. S., Anderson, R. C., & Nisbet, D. J. (2009). Diet, *Escherichia coli* O157:H7, and cattle: a review after 10 years. *Current Issues in Molecular Biology*, *11*, 67−79.

Carlton, R. M., Noordman, W. H., Biswas, B., De Meester, E. D., & Loessner, M. J. (2005). Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology*, *43*, 301−312.

Carroll, C. (2009). Cadbury's *Salmonella* scare: good or bad crisis management? In A. Lindgreen, M. K. Hingley, & J. Vanhamme (Eds.), *The crisis of food brands: Sustaining safe, innovative, and competitive food supply* (pp. 15−28).

Carter, C. D., Parks, A., Abuladze, T., Li, M., Woolston, J., Magnone, J., et al. (2012). Bacteriophage cocktail significantly reduces *Escherichia coli* O157: H7 contamination of lettuce and beef, but does not protect against recontamination. *Bacteriophage*, *2*, 178−185.

Cavallaro, E., Date, K., Medus, C., Meyer, S., Miller, B., Kim, C., et al. (2011). *Salmonella typhimurium* infections associated with peanut products. *New England Journal of Medicine*, *365*, 601−610.

CDC. (2010). *Investigation update: Multistate outbreak of human* Salmonella enteritidis *infections associated with shell eggs* [Online]. Atlanta, GA: CDC, U.S. Department of Health and Human Services. Available http://www.cdc.gov/salmonella/enteritidis/, 2013.

Cerveny, K. E., Depaola, A., Duckworth, D. H., & Gulig, P. A. (2002). Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infection and Immunity*, *70*, 6251−6262.

Chai, Y., Li, S., Horlikawa, S., Park, M.-K., Vodyanoy, V., & Chin, B. A. (2012). Rapid and sensitive detection of *Salmonella typhimurium* on eggshells by using wireless biosensors. *Journal of Food Protection*, *75*, 631−636.

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiology*, *8*, 769−783.

Chibeu, A., Agius, L., Gao, A., Sabour, P. M., Kropinski, A. M., & Balamurugan, S. (2013). Efficacy of bacteriophage LISTEX™P100 combined with chemical antimicrobials in reducing *Listeria monocytogenes* in cooked turkey and roast beef. *International Journal of Food Microbiology*, *167*, 208−214.

Coffey, B., Mills, S., Coffey, A., Mcauliffe, O., & Ross, R. P. (2010). Phage and their lysins as biocontrol agents for food safety applications. *Annual Review of Food Science and Technology*, *1*, 449−468.

Connerton, P. L., Timms, A. R., & Connerton, I. F. (2011). *Campylobacter* bacteriophages and bacteriophage therapy. *Journal of Applied Microbiology*, *111*, 255−265.

Crothers-Stomps, C., Høj, L., Bourne, D. G., Hall, M. R., & Owens, L. (2010). Isolation of lytic bacteriophage against *Vibrio harveyi*. *Journal of Applied Microbiology*, *108*, 1744−1750.

D'Aoust, J. Y. (1994). *Salmonella* and the international food trade. *International Journal of Food Microbiology*, *24*(1−2), 11−31.

D'Aoust, J. Y. (2001). Salmonella. In R. G. Labbé, & S. Garcíia (Eds.), *Guide to foodborne pathogens*. New York: John Wiley and Sons.

Davis, M. A., Besser, T. E., Eckmann, K., Macdonald, J. K., Green, D., Hancock, D. D., et al. (2007). Multidrug-resistant *Salmonella typhimurium*, Pacific Northwest, United States. *Emerging Infectious Diseases*, *13*(10). Available from http://wwwnc.cdc.gov/eid/article/13/10/07-0536.htm, 20 December 2013.

Debroy, C., Roberts, E., Valadez, A. M., Dudley, E. G., & Cutter, C. N. (2011). Detection of shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the *wzx* gene of the O-antigen gene cluster. *Foodborne Pathogen Disease*, *8*, 651−652.

Donnelly, C. W. (2001). *Listeria monocytogenes*: a continuing challenge. *Nutrition Reviews*, *59*, 183−194.

Doré, K., Buxton, J., Henry, B., Pollari, F., Middleton, D., Fyfe, M., et al. (2004). Risk factors for *Salmonella* Typhimurium DT 104 and non-DT 104 infection: a Canadian multi-provincial case-control study. *Epidemiology and Infection*, *132*, 485−493.

Dykes, G. A., & Moorhead, S. M. (2002). Combined antimicrobial effect of nisin and a listeriophage against *Listeria monocytogenes* in broth but not in buffer or on raw beef. *International Journal of Food Microbiology*, *73*, 71−81.

Dziejman, M., & Yildiz, F. H. (2011). Genomics of pathogenic *Vibrio* species. In M. Wiedmann, & W. Zhang (Eds.), *Genomics of foodborne bacterial pathogens*. New York: Springer.

Ehling-Schulz, M., Fricker, M., & Scherer, S. (2004). *Bacillus cereus*, the causative agent of an emetic type of foodborne illness. *Molecular Nutrition & Food Research*, *48*, 479−487.

El-Arabi, T. (2011). *Application of specific lytic bacteriophages to control Bacillus cereus cells and spores in milk* (Ph.D.). University of Guelph.

El-Arabi, T. F., Griffiths, M. W., She, Y.-M., Villegas, A., Lingohr, E. J., & Kropinski, A. M. (2013). Genome sequence and analysis of a broad-host range lytic bacteriophage that infects the *Bacillus cereus* group. *Virology Journal*, *10*(1), 48.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P., & Connerton, I. (2009). Application of a group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *Journal of Food Protection*, *72*, 733−740.

Farber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes*, a foodborne pathogen. *Microbiology and Molecular Biology Reviews*, *55*, 476−511.

Farmer, J. J., Asbury, M. A., Hickman, F. W., & Brenner, D. J. (1980). *Enterobacter sakazakii* − a new species of "enterobacteriaceae" isolated from clinical specimens. *International Journal of Systematic Bacteriology*, *30*, 569−584.

Feng, P. (1995). *Escherichia coli* serotype O157:H7: novel vehicles of infection and emergence of phenotypic variants. *Emerging Infectious Diseases*, *1*(2), 47−52.

Fields, P. I., Popovic, T., Wachsmuth, K., & Olsvik, O. (1992). Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. *Journal of Clinical Microbiology*, *30*, 2118−2121.

Fiorentin, L., Vieira, N. D., & Barioni Júnior, W. (2005). Use of lytic bacteriophages to reduce *Salmonella enteritidis* in experimentally contaminated chicken cuts. *Revista Brasileira de Ciência Avícola*, *7*, 255−260.

Fischetti, V. (2005). The use of phage lytic enzymes to control bacterial infections. In E. Kutter, & A. Sulakvelidze (Eds.), *Bacteriophages: Biology and applications*. Boca Raton, FL: CRC Press.

Fujinaga, Y., Sugawara, Y., & Matsumura, T. (2013). Uptake of botulinum neurotoxin in the intestine. In A. Rummel, & T. Binz (Eds.), *Botulinum neurotoxins*. Berlin Heidelberg: Springer.

Galvez, A., Abriouel, H., Benomar, N., & Lucas, R. (2010). Microbial antagonists to foodborne pathogens and biocontrol. *Current Opinion in Biotechnology*, *21*, 142−148.

Garcia, P., Martinez, B., Obeso, J. M., & Rodriguez, A. (2008). Bacteriophages and their application in food safety. *Letters in Applied Microbiology*, *47*, 479−485.

Garcia, P., Rodriguez, L., Rodriguez, A., & Martinez, B. (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science & Technology*, *21*, 373−382.

Gaynor, K., Park, S. Y., Kanenaka, R., Colindres, R., Mintz, E., Ram, P. K., et al. (2009). International foodborne outbreak of *Shigella sonnei* infection in airline passengers. *Epidemiology and Infection*, *137*, 335−341.

Goode, D., Allen, V. M., & Barrow, P. A. (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Applied and Environmental Microbiology*, *69*, 5032−5036.

Gosney, M. A., Martin, M. V., Wright, A. E., & Gallagher, M. (2006). *Enterobacter sakazakii* in the mouths of stroke patients and its association with aspiration pneumonia. *European Journal of Internal Medicine*, *17*, 185−188.

Gould, L. H., & Limbago, B. (2010). *Clostridium difficile* in food and domestic animals: a new foodborne pathogen? *Clinical Infectious Diseases*, *51*, 557−582.

Graves, L. M., Helsel, L. O., Steigerwalt, A. G., Morey, R. E., Daneshvar, M. I., Roof, S. E., et al. (2010). *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 1280−1288.

Griffiths, P. L., & Park, R. W. A. (1990). Campylobacters associated with human diarrheal disease. *Journal of Applied Bacteriology*, *69*, 281−301.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., & Loessner, M. J. (2012). Biocontrol of *Salmonella typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. *International Journal of Food Microbiology*, *154*, 66−72.

Guenther, S., Huwyler, D., Richard, S., & Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and Environmental Microbiology*, *75*, 93−100.

Guh, A., Phan, Q., Randall, N., Purviance, K., Milardo, E., Kinney, S., et al. (2010). Outbreak of *Escherichia coli* O157 associated with raw milk, Connecticut, 2008. *Clinical Infectious Diseases*, *51*, 1411−1417.

Gulig, P. A., Bourdage, K. L., & Starks, A. M. (2005). Molecular pathogenesis of *Vibrio vulnificus*. *Journal of Microbiology*, *43*, 118−131.

Gurtler, J. B., Kornacki, J. L., & Beuchat, L. R. (2005). *Enterobacter sakazakii*: a coliform of increased concern to infant health. *International Journal of Food Microbiology*, *104*, 1−34.

Hagens, S., & Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Applied Microbiology and Biotechnology*, *76*, 513−519.

Hagens, S., & Loessner, M. J. (2010). Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Current Pharmaceutical Biotechnology*, *11*, 58−68.

Haq, I. U., Chaudhry, W. N., Akhtar, M. N., Andleeb, S., & Qadri, I. (2012). Bacteriophages and their implications on future biotechnology: a review. *Virology Journal*, *9*, 9.

Hartland, E. L., & Leong, J. (2013). Enteropathogenic and enterohemorrhagic *E. coli*: ecology, pathogenesis and evolution. *Frontiers in Cellular and Infection Microbiology*, *3*, 15.

Healy, B., Cooney, S., O'brien, S., Iversen, C., Whyte, P., Nally, J., et al. (2010). *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. *Foodborne Pathogens and Disease*, *7*, 339−350.

Hertwig, S., Hammerl, J. A., Appel, B., & Alter, T. (2013). Post-harvest application of lytic bacteriophages for biocontrol of foodborne pathogens and spoilage bacteria. *Berliner Und Munchener Tierarztliche Wochenschrift*, *126*, 357−369.

Hibma, A. M., Jassim, S. A. A., & Griffiths, M. W. (1997). Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. *International Journal of Food Microbiology*, *34*, 197−207.

Hooton, S. P. T., Atterbury, R. J., & Connerton, I. F. (2011). Application of a bacteriophage cocktail to reduce *Salmonella* Typhimurium U288 contamination on pig skin. *International Journal of Food Microbiology*, *151*, 157−163.

Hornstra, L. M., De Leeuw, P. L. A., Moezelaar, R., Wolbert, E. J., De Vries, Y. P., De Vos, W. M., et al. (2007). Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology*, *116*, 367−371.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., et al. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter genomospecies* I. *BMC Evolutionary Biology*, *7*, 64.

Jassim, S. A. A., Abdulamir, A. S., & Abu Bakar, F. (2012). Novel phage-based bio-processing of pathogenic *Escherichia coli* and its biofilms. *World Journal of Microbiology and Biotechnology*, *28*, 47−60.

Jawa, R. S., & Mercer, D. W. (2012). *Clostridium difficile*−associated infection: a disease of varying severity. *American Journal of Surgery*, *204*, 836−842.

Jay, J. M. (2000). *Modern food microbiology*. Gaithersburg, MD: Aspen Publishers.

Jay, J.M., Loessner, M.J. & Golden, D.A. (2005). Food poisoning caused by Gram positive sporeforming bacteria. Modern food microbiology. Springer US.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., & Forsythe, S. J. (2011). *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. *genomospecies* 1, recovered from a leg infection, water, and food ingredients. *International Journal of Systematic and Evolutionary Microbiology*, *62*, 1277−1283.

Jørgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D. R. A., Bolton, F. J., et al. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *International Journal of Food Microbiology*, *76*, 151−164.

Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel natural food antimicrobials. In M. P. Doyle, & T. R. Klaenhammer (Eds.), *Annual review of food science and technology* (vol. 3). Palo Alto: Annual Reviews.

Kang, H. W., Kim, J. W., Jung, T. S., & Woo, G. J. (2013). wksl3, a New biocontrol agent for *Salmonella enterica* serovars Enteritidis and Typhimurium in foods: characterization, application, sequence analysis, and oral acute toxicity study. *Applied and Environmental Microbiology*, *79*, 1956−1968.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, *2*, 123−140.

Karunasagar, I., Shivu, M. M., Girisha, S. K., & Krohne, G. (2007). Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture*, *268*, 288−292.

Kim, K. P., Klumpp, J., & Loessner, M. J. (2007). *Enterobacter sakazakii* bacteriophages can prevent bacterial growth in reconstituted infant formula. *International Journal of Food Microbiology*, *115*, 195−203.

Kim, T. J., Weng, W. L., Silva, J. L., Jung, Y. S., & Marshall, D. (2010). Identification of natural antimicrobial substances in red muscadine juice against *Cronobacter sakazakii*. *Journal of Food Science*, *75*, M150−M154.

Kocharunchitt, C., Ross, T., & Mcneil, D. L. (2009). Use of bacteriophages as biocontrol agents to control *Salmonella* associated with seed sprouts. *International Journal of Food Microbiology*, *128*, 453−459.

Kothary, M. H., & Babu, U. S. (2001). Infective dose of foodborne pathogens in volunteers: a review. *Journal of Food Safety*, *21*, 49−73.

Kutateladze, M., & Adamia, R. (2008). Phage therapy experience at the Eliava Institute. *Médecineet Maladies Infectieuses*, *38*, 426−430.

Kutateladze, M., & Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, *28*, 591−595.

Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., et al. (2010). Phage therapy in clinical practice: treatment of human infections. *Current Pharmaceutical Biotechnology*, *11*(18), 69−86.

Lacroix, C. (2011). *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation*. Woodhead Publishing.

Lang Halter, E., Neuhaus, K., & Scherer, S. (2013). *Listeria weihenstephanensis* sp. nov., isolated from the water plant *Lemna trisulca* of a German fresh water pond. *International Journal of Systematic and Evolutionary Microbiology*, *63*(Pt 2), 641−647. http://dx.doi.org/10.1099/ijs.0.036830-0.

Leclercq, A., Clermont, D., Bizet, C., Grimont, P. A. D., Le Fleche-Mateos, A., Roche, S. M., et al. (2010). *Listeria rocourtiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 2210−2214.

Lee, J.-H., Shin, H., Son, B., & Ryu, S. (2012). Complete genome sequence of *Bacillus cereus* bacteriophage BCP78. *Journal of Virology*, *86*, 637−638.

Lee, W. J., Billington, C., Hudson, J. A., & Heinemann, J. A. (2011). Isolation and characterization of phages infecting *Bacillus cereus*. *Letters in Applied Microbiology*, *52*, 456−464.

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., et al. (2001). Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *Journal of Food Protection*, *64*(6), 1116−1121.

Loc Carrillo, C., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Applied and Environmental Microbiology*, *71*, 6554−6563.

Loessner, M. J., Maier, S. K., Daubek-Puza, H., Wendlinger, G., & Scherer, S. (1997). Three *Bacillus cereus* bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different bacilli. *Journal of Bacteriology*, *179*, 2845−2851.

Loos, S., Ahlenstiel, T., Kranz, B., Staude, H., Pape, L., Härtel, C., et al. (2012). An outbreak of shiga-toxin producing *E. coli* O104:H4 hemolytic uremic syndrome (STEC-HUS) in Germany: presentation and short-term outcome in children. *Clinical Infectious Diseases*, *55*, 753−759.

López-Cuevas, O., Campo-Del Campo, N., León-Félix, J., Valdez-Torres, B., & Chaidez, C. (2012). Evaluation of bacteriophage av-08 for simultaneous biocontrol of *Salmonella montevideo* and *Escherichia coli* O157:H7 in experimentally contaminated chicken skin. *Journal of Food Safety*, *32*, 305−310.

Mandrell, R. E., Harden, L. A., Bates, A., Miller, W. G., Haddon, W. F., & Fagerquist, C. K. (2005). Speciation of *Campylobacter coli*, *C. jejuni*, *C. helveticus*, *C. lari*, *C. sputorum*, and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology*, *71*, 6292−6307.

Mayer, M. J., Narbad, A., & Gasson, M. J. (2008). Molecular characterization of a *Clostridium difficile* bacteriophage and its cloned biologically active endolysin. *Journal of Bacteriology*, *190*, 6734−6740.

Meader, E., Mayer, M. J., Gasson, M. J., Steverding, D., Carding, S. R., & Narbad, A. (2010). Bacteriophage treatment significantly reduces viable *Clostridium difficile* and prevents toxin production in an in vitro model system. *Anaerobe*, *16*, 549−554.

Medus, C., Meyer, S., Smith, K., Jawahir, S., Miller, B., Viger, K., et al. (2009). Multistate outbreak of *Salmonella* infections associated with peanut butter and peanut butter-containing products − United States, 2008−2009. *Morbidity and Mortality Weekly Report*, *58*, 85−90.

Merril, C. R., Scholl, D., & Adhya, S. L. (2003). The prospect for bacteriophage therapy in western medicine. *Nature Reviews Drug Discovery*, *2*, 489−497.

Miller, R. W., Skinner, J., Sulakvelidze, A., Mathis, G. F., & Hofacre, C. L. (2010). Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with *Clostridium perfringens*. *Avian Diseases*, *54*, 33−40.

Modi, R., Hirvi, Y., Hill, A., & Griffiths, M. W. (2001). Effect of phage on survival of *Salmonella enteritidis* during manufacture and storage of cheddar cheese made from raw and pasteurized milk. *Journal of Food Protection*, *64*, 927−933.

Moffatt, C. R., & Musto, J. (2013). *Salmonella* and egg-related outbreaks. *Microbiology Australia*, *34*, 94−98.

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., & Harper, D. R. (2010). Bacteriophage applications: where are we now? *Letters in Applied Microbiology*, *51*, 363−369.

Montañez-Izquierdo, V. Y., Salas-Vázquez, D. I., & Rodríguez-Jerez, J. J. (2012). Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling *Listeria monocytogenes* biofilms on stainless steel surfaces. *Food Control*, *23*, 470−477.

Mullane, N. R., Iversen, C., Healy, B., Walsh, C., Whyte, P., Wall, P. G., et al. (2007). *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. *Minerva Pediatrica*, *59*, 137−148.

O'Flaherty, S., Ross, R. P., & Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS Microbiology Reviews*, *33*, 801−819.

O'Flynn, G., Ross, R. P., Fitzgerald, G. F., & Coffey, A. (2004). Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, *70*, 3417−3424.

Oliveira, H., Azeredo, J., Lavigne, R., & Kluskens, L. D. (2012). Bacteriophage endolysins as a response to emerging foodborne pathogens. *Trends in Food Science & Technology*, *28*, 103−115.

Oliveira, J., Castilho, F., Cunha, A., & Pereira, M. J. (2012). Bacteriophage therapy as a bacterial control strategy in aquaculture. *Aquaculture International*, *20*, 879−910.

Patel, J., Sharma, M., Millner, P., Calaway, T., & Singh, M. (2011). Inactivation of *Escherichia coli* O157:H7 attached to spinach harvester blade using bacteriophage. *Foodborne Pathogens and Diseases*, *8*, 541−546.

Phumkhachorn, P., & Rattanachaikunsopon, P. (2010). Isolation and partial characterization of a bacteriophage infecting the shrimp pathogen *Vibrio harveyi*. *African Journal of Microbiology Research*, *4*, 1794−1800.

Priest, F. G., Barker, M., Baillie, L. W. J., Holmes, E. C., & Maiden, M. C. J. (2004). Population structure and evolution of the *Bacillus cereus* group. *Journal of Bacteriology*, *186*, 7959−7970.

Quintero, M., Maldonado, M., Perez-Munoz, M., Jimenez, R., Fangman, T., Rupnow, J., et al. (2011). Adherence inhibition of *cronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. *Current Microbiology*, *62*, 1448−1454.

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., et al. (2011). Origins of the *E. coli* strain causing an outbreak of hemolytic−uremic syndrome in Germany. *New England Journal of Medicine*, *365*, 709−717.

Rees, C. E. D., & Dodd, C. E. R. (2006). Phage for rapid detection and control of bacterial pathogens in food. *Advances in Applied Microbiology*, *59*, 159−186.

Rees, C. E. D., & Loessner, M. J. (2005). Phage for the detection of pathogenic bacteria. In E. Kutter, & A. Sulakvelidze (Eds.), *Bacteriophage: Biology and applications*. Boca Raton, FL: CRC Press.

Reiss, G., Kunz, P., Koin, D., & Keeffe, E. B. (2006). *Escherichia coli* O157:H7 infection in nursing homes: review of literature and report of recent outbreak. *Journal of the American Geriatrics Society*, *54*, 680−684.

Rocourt, J., & Buchrieser, C. (2007). The genus *Listeria* and *Listeria monocytogenes*: phylogenetic position, taxonomy, and identification. In E. T. Ryser, & E. H. Marth (Eds.), *Listeria, listeriosis, and food safety* (3rd ed.). Boca Raton, FL: CRC Press.

Rohde, H., Qin, J., Cui, Y., Li, D., Loman, N. J., Hentschke, M., et al. (2011). Open-source genomic analysis of shiga-toxin−producing *E. coli* O104:H4. *New England Journal of Medicine*, *365*, 718−724.

Roy, B., Ackermann, H. W., Pandian, S., Picard, G., & Goulet, J. (1993). Biological inactivation of adhering *Listeria monocytogenes* by listeriaphages and a quaternary ammonium compound. *Applied and Environmental Microbiology*, *59*, 2914−2917.

Sabour, P. M., & Griffiths, M. W. (2010). *Bacteriophages in the control of food- and waterborne pathogens*. Washington, DC: ASM Press.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases*, *17*, 7−15.

Schmelcher, M., Donovan, D. M., & Loessner, M. J. (2012). Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, *7*, 1147−1171.

Scholl, D., Rogers, S., Adhya, S., & Merril, C. R. (2001). Bacteriophage K1-5 encodes two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of *Escherichia coli*. *Journal of Virology*, *75*, 2509−2515.

Seal, B. S. (2013). Characterization of bacteriophages virulent for *Clostridium perfringens* and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium. *Poultry Science*, *92*, 526−533.

Seckin, A. K., & Baladura, E. (2010). Bacteriocin and bacteriophage applications in foods preservation. *Gida*, *35*, 461−467.

See, K. C., Than, H. A., & Tang, T. (2007). *Enterobacter sakazakii* bacteraemia with multiple splenic abscesses in a 75-year-old woman: a case report. *Age and Ageing*, *36*, 595−596.

Shane, S. M. (2000). *Campylobacter* infection of commercial poultry. *Revue Scientifique et Technique*, *19*, 376−395.

Sharma, M., & Goodridge, L. (2013). Bacteriophages: back to the future. *Food Technology*, *67*, 46−55.

Sharma, M., Patel, J. R., Conway, W. S., Ferguson, S., & Sulakvelidze, A. (2009). Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. *Journal of Food Protection*, *72*, 1481−1485.

Shivu, M. M., Rajeeva, B. C., Girisha, S. K., Krohne, G., & Karunasagar, I. (2007). Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Environmental Microbiology*, *9*, 322−331.

Sillankorva, S. M., Oliveira, H., & Azeredo, J. (2012). Bacteriophages and their role in food safety. *International Journal of Microbiology*, *2012*, 863945.

Silva, R. O. S., Ribeiro, M. G., Palhares, M. S., Borges, A. S., Maranhão, R. P. A., Silva, M. X., et al. (2013). Detection of A/B toxin and isolation of *Clostridium difficile* and *Clostridium perfringens* from foals. *Equine Veterinary Journal*, *45*, 671−675.

Siringan, P., Connerton, P. L., Payne, R. J. H., & Connerton, I. F. (2011). Bacteriophage-mediated dispersal of *Campylobacter jejuni* biofilms. *Applied and Environmental Microbiology*, *77*, 3320−3326.

Sivapalasingam, S., Nelson, J. M., Joyce, K., Hoekstra, M., Angulo, F. J., & Mintz, E. D. (2006). High prevalence of antimicrobial resistance among *Shigella* isolates in the United States tested by the National Antimicrobial Resistance Monitoring System from 1999 to 2002. *Antimicrobial Agents and Chemotherapy*, *50*, 49−54.

Songer, J. G. (2004). The emergence of *Clostridium difficile* as a pathogen of food animals. *Animal Health Research Reviews*, *5*, 321−326.

Soni, K., Nannapaneni, R., & Hagens, S. (2010). Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophages Listex P100. *Foodborne Pathogens and Disease*, *7*, 427−434.

Srinivasan, P., Ramasamy, P., Brennan, G. P., & Hanna, R. E. B. (2007). Inhibitory effects of bacteriophages on the growth of *Vibrio* sp., pathogens of shrimp in the Indian aquaculture environment. *Asian Journal of Animal and Veterinary Advances*, *2*, 166−183.

Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, *32*, 579−606.

Sulakvelidze, A. (2013). Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. *Journal of the Science of Food and Agriculture*, *93*, 3137−3146.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G., Jr. (2001). Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy*, *45*, 649−659.

Sulakvelidze, A., & Barrow, P. (2005). Phage therapy in animals and agribusiness. In E. Kutter, & A. Sulakvelidze (Eds.), *Bacteriophages: Biology and application*. Boca Raton, FL: CRC Press.

Sumner, J., & Ross, T. (2002). A semi-quantitative seafood safety risk assessment. *International Journal of Food Microbiology*, *77*, 55−59.

Sutherland, J., & Varnam, A. (2002). Enterotoxin-producing *Staphylococcus*, *Shigella*, *Yersinia*, *Vibrio*, *Aeromonas* and *Plesiomonas*. In C. W. Blackburn, & P. J. McClure (Eds.), *Foodborne pathogens: Hazards, risk analysis and control*. Boca Raton, FL: CRC Press.

Swaminathan, B., & Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection*, *9*, 1236−1243.

Ting, P. T., & Freiman, A. (2004). The story of *Clostridium botulinum*: from food poisoning to botox. *Clinical Medicine*, *4*, 258−261.

Todd, E. C. D., & Notermans, S. (2011). Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control*, *22*, 1484−1490.

Tourasse, N. J., Helgason, E., Økstad, O. A., Hegna, I. K., & Kolstø, A. B. (2006). The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. *Journal of Applied Microbiology*, *101*, 579−593.

Turki, Y., Ouzari, H., Mehri, I., Ammar, A. B., & Hassen, A. (2012). Evaluation of a cocktail of three bacteriophages for the biocontrol of *Salmonella* of wastewater. *Food Research International*, *45*, 1099−1105.

Van Opstal, I., Bagamboula, C. F., Vanmuysen, S. C. M., Wuytack, E. Y., & Michiels, C. W. (2004). Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *International Journal of Food Microbiology*, *92*, 227−234.

Viazis, S., Akhtar, M., Feirtag, J., & Diez-Gonzalez, F. (2011). Reduction of *Escherichia coli* O157:H7 viability on hard surfaces by treatment with a bacteriophage mixture. *International Journal of Food Microbiology*, *145*, 37−42.

Vinod, M. G., Shivu, M. M., Umesha, K. R., Rajeeva, B. C., Krohne, G., Karunasagar, I., et al. (2006). Isolation of *Vibrio harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. *Aquaculture*, *255*, 117−124.

Wagenaar, J. A., Van Bergen, M. A. P., Mueller, M. A., Wassenaar, T. M., & Carlton, R. M. (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Veterinary Microbiology*, *109*, 275−283.

Wahl, E., Rømma, S., & Granum, P. E. (2013). A *Clostridium perfringens* outbreak traced to temperature-abused beef stew, Norway, 2012. *EuroSurveillance*, *18*(9). pii=20408.

Walter, M. H. (2003). Efficacy and durability of *Bacillus anthracis* bacteriophages used against spores. *Journal of Environmental Health*, *66*, 9.

Whichard, J. M., Namalwar, S., & Pierson, F. W. (2003). Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters. *Journal of Food Protection*, *66*(6), 220−225.

Willshaw, G., Cheasty, T., & Smith, H. R. (2000). Escherichia coli. In B. M. Lund, T. C. Baird-Parker, & G. W. Gould (Eds.), *The microbiological safety and quality of food*. Maryland, USA: Aspen Inc.

Wittman, R., & Flick, G. (1995). Microbial contamination of shellfish: prevalence, risk to human health, and control strategies. *Annual Review of Public Health*, *16*, 123−140.

Yan, Q. Q., Condell, O., Power, K., Butler, F., Tall, B. D., & Fanning, S. (2012). *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium. *Journal of Applied Microbiology*, *113*, 1−15.

Yoong, P., Schuch, R., Nelson, D., & Fischetti, V. A. (2006). PlyPH, a bacteriolytic enzyme with a broad pH range of activity and lytic action against *Bacillus anthracis*. *Journal of Bacteriology*, *188*, 2711−2714.

Young, C. R., Ziprin, R. L., Hume, M. E., & Stanker, L. H. (1999). Dose response and organ invasion of day-of-hatch Leghorn chicks by different isolates of *Campylobacter jejuni*. *Avian Diseases*, *43*, 763−767.

Yu, R., Wang, S., Yu, Y.-Z., Du, W.-S., Yang, F., Yu, W.-Y., et al. (2009). Neutralizing antibodies of botulinum neurotoxin serotype a screened from a fully synthetic human antibody phage display library. *Journal of Biomolecular Screening*, *14*, 991−998.

Zdanovsky, A., & Zdanovskaia, M. (2013). Recombinant proteins that trigger production of antibodies recognizing botulinum neurotoxin while not possessing sequences of this toxin. *Toxicon*, *67*, 63−70.

Zhang, H., Wang, H., & Bao, H. (2013). Phage inactivation of foodborne *Shigella* on ready-to-eat spiced chicken. *Poultry Science*, *92*, 211−217.

Zhou, G., Liu, H., He, J., Yuan, Y., & Yuan, Z. (2008). The occurrence of *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *International Journal of Food Microbiology*, *121*, 195−200.

Zuber, S., Boissin-Delaporte, C., Michot, L., Iversen, C., Diep, B., Bruessow, H., et al. (2008). Decreasing *Enterobacter sakazakii(Cronobacter* spp.) food contamination level with bacteriophages: prospects and problems. *Microbial Biotechnology*, *1*, 532−543.

Lactic acid bacteria (LAB) as antimicrobials in food products: types and mechanisms of action

6

C.A. O'Bryan[1], P.G. Crandall[1], S.C. Ricke[1], J.B. Ndahetuye[2]
[1]University of Arkansas, Fayetteville, AR, USA; [2]Institute of Agriculture and Animal Husbandry, Kigali, Africa

6.1 Introduction

The first use of lactic acid bacteria (LAB) to preserve foods has been lost in antiquity, although there are indications that fermented meats, milk, and vegetables were made 6000 years ago (Fox, 1993; Ricke, Koo, & Keeton, 2013). It is believed that the Chinese coolies working on the Great Wall of China in the third century BC had fermented vegetables as part of their diet (Pederson, 1979). These ancient peoples would not have had an appreciation for the role of LAB in the preservation process; nevertheless they developed traditional means of handling and storing foods in a prescribed manner that would increase the keeping time and taste of the original food matrix. They did understand the need for an inoculum and satisfied this requirement by keeping a portion of their previously fermented material as a starter for new fermentations (Hansen, 2002). It was not until the development of microbiology in the 1850s that the role of microorganisms in fermentation was discovered. In 1857 Louis Pasteur discovered the role of LAB in the spoilage of wine (Wibowo, Eschenbruch, Davis, Fleet, & Lee, 1985), and in 1873 Joseph Lister was able to isolate a pure culture of a lactic acid-producing bacterium (König & Fröhlich, 2009).

Calculating, with any degree of certainty, the long-term economic benefits, alleviation of human suffering from food-borne illness, food products developed, and increased consumers' enjoyment would be a monumental undertaking, but it suffices to say LAB have contributed to the viability of the food industry and across the food safety spectrum. In this chapter we focus on the characteristics of LAB, the effects of culture preparation and storage on LAB characteristics, and the antimicrobial compounds produced by LAB.

6.2 Characteristics of lactic acid bacteria (LAB)

Typical LAB are Gram-positive, non-spore-forming rods or cocci producing lactic acid as the major end product of fermentation of glucose (Vandamme et al., 1996). There have been frequent changes in the taxonomic classification of LAB, but most

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classification schemes agree that genera in the order Lactobacillales, which includes *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, in addition to *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*, are the genera of LAB (Stiles & Holzapfel, 1997; Vandamme et al., 1996). Some authors have included *Bifidobacterium* in this group because it has probiotic activity, but these bacteria actually are genetically distinct from the LAB (Vandamme et al., 1996).

Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative (Axelsson, 1998). The main products of fermentation include organic acids, alcohol, and carbon dioxide (Ray & Daeschel, 1992), although LAB may also produce aromatic molecules, vitamins, or bioactive peptides (Ross, Morgan, & Hill, 2002).

LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources and are therefore dependent on preformed amino acids being present in the growth medium; this requirement for amino acids differs among species and strains within species, with some strains requiring as many as 15 preformed amino acids (Dunn, Shankman, Camien, & Block, 1947). Because free amino acids are not typically present in sufficient concentrations in the environment, the LAB require a proteolytic system capable of hydrolyzing peptides and proteins to obtain their essential amino acids (Fernández & Zúñiga, 2006). The proteolytic activity of LAB contributes additionally to the development of the flavor, aroma, and texture of fermented products; for instance, many varieties of cheeses, such as Swiss and Cheddar, rely on proteolysis for their desirable flavor notes (Fernández & Zúñiga, 2006).

6.3 Carbohydrate metabolism in LAB

As a group the LAB are capable of degrading many different carbohydrates and related compounds. Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative.

Homofermentative LAB ferment hexoses to lactic acid by the Embden−Meyerhof (E−M) pathway; this group includes some lactobacilli and most species of enterococci, lactococci, pediococci, and streptococci (Kandler, 1983). Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize 1 mol of glucose in the E−M pathway to yield 2 mol of pyruvate; nicotinamide adenine dinucleotide (NADH) is oxidized simultaneously with pyruvate reduction to yield 2 mol of lactic acid and 2 mol of ATP per mole glucose consumed (Kandler, 1983).

Heterofermentative LAB use the phosphoketolase pathway (pentose−phosphate pathway) to break down sugars. In this pathway 1 mol of glucose is broken down into equimolar amounts of ethanol and ATP. The heterofermentative LAB include leuconostocs and some lactobacilli. Figure 6.1 illustrates these metabolic patterns.

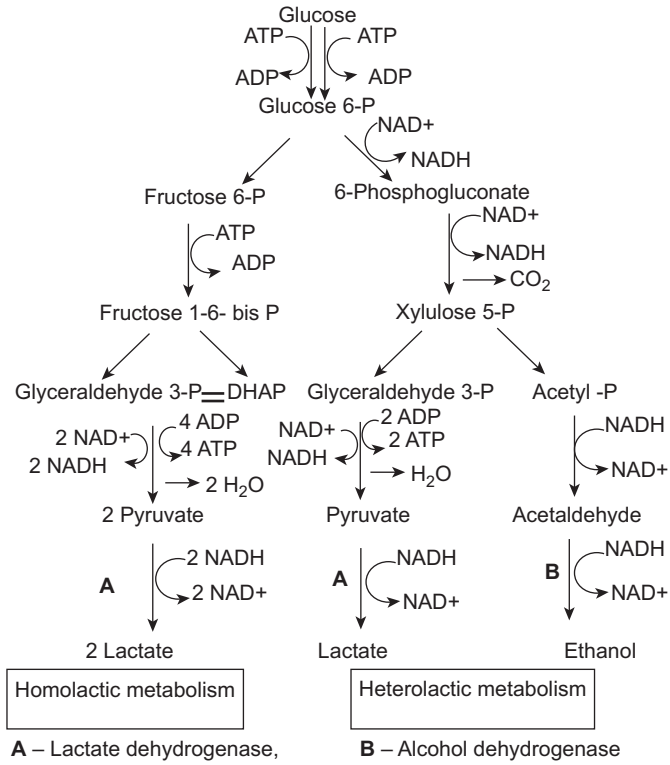

Figure 6.1 Metabolism of lactic acid bacteria.
Source: From Reddy, Altaf, Naveena, Venkateshwar, and Vijay Kumar (2008).

6.4 Effects of culture preparation and storage techniques on LAB

LAB are widely used as starter cultures for manufacturing cheeses and fermented products (milk, meats, vegetables, and bread), and several species have been shown to exhibit beneficial effects on human health. The use of LAB as starter cultures for food fermentations and use as probiotic cultures depends strongly on the preservation technologies employed, which are required to ensure shelf-stable cultures in terms of viability and activity. The preparation of starter cultures and probiotics requires production and maintenance techniques that maximize viability, activity, and storage stability of bacterial cells. Various methods of preservation may be used for LAB, including spray drying, freezing, or freeze drying (Broadbent & Lin, 1999).

6.4.1 Effects of freezing on LAB

The ability of LAB to survive freezing and frozen storage differs not only between species of an organism but also among strains of a single species (Morichi, Irie,

Yano, & Kembo, 1963; Picque, Perret, Latrille, & Corrieu, 1992; Tsvetkov & Shishkova, 1982). Other factors that influence survival of LAB are the culture conditions before freezing, method of harvesting cells, type and concentration of cryoprotectant, freezing conditions, and final storage temperature (Fonseca, Béal, & Corrieu, 2001). In a study of the effects of experimental factors on the resistance of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* to freezing and frozen storage, Fonseca et al. (2001) found that of all their test variables only the thawing temperature after freezing did not produce any significant impact on viability of cells or acidification activity after freezing.

Cells were frozen either overnight at −70 °C or immediately by direct immersion in liquid nitrogen (−196 °C). They found that *Str. thermophilus* was more resistant than *Lactobacillus bulgaricus*, confirmed that glycerol addition to the medium during freezing and storage had a cryoprotective effect, and demonstrated that freezing at a lower temperature (−136 °C) for a shorter time and a low storage temperature (−70 °C as opposed to −20 °C) were more protective of the recovery of LAB (Fonseca et al., 2001). Damage to cells at low freezing rates has been speculated to be caused by osmotic stress to cells when the formation of extracellular ice induces high solute concentrations in the extracellular medium, exposing the cells to increased ionic concentrations and changes in pH for extended periods of time (Mazur, 1966). At very rapid cooling rates, it has been assumed that LAB cellular damage is due to the formation of intracellular ice (Karlsson, Cravalho, & Toner, 1993; Mazur, 1977). However, Fonseca, Marin, and Morris (2006) studied the interactions between freezing kinetics and subsequent storage temperatures and their effects on the biological activity of LAB. The viability and acidification activity of *La. delbrueckii* subsp. *bulgaricus* CFL1 after storage at low temperatures (−20 °C and −80 °C) in the presence of glycerol decreased after freezing and were strongly dependent on freezing kinetics, with high cooling rates resulting in minimum loss. The viability and acidification activity after freezing were greatest at a very high cooling rate (2500 °C/min (immersion in liquid nitrogen)). A low storage temperature (−80 °C) maintained viability and acidification activity after storage for 1 month regardless of freezing method. For samples stored at −20 °C, the maximum loss of viability and acidification activity was observed with cells that had been rapidly cooled (immersed directly in liquid nitrogen). When observed by scanning electron microscopy, these cells did not contain intracellular ice, and they were observed to have the plasma membrane pulled away from the cell wall, suggesting that the cell damage was caused by an osmotic imbalance during warming, not the formation of intracellular ice during freezing (Fonseca et al., 2006). Thus, viability and acidification activity after freezing will be greatest at a very high cooling rate and a low storage temperature (−80 °C); however, low cooling rates are preferable if a high storage temperature (−20 °C) is used.

6.4.2 Effects of freeze drying on LAB

Fonseca, Béal, and Corrieu (2000) reported that enterococci (i.e., small spherical cells) are apparently more resistant to freeze drying than longer rod-shaped lactobacilli; this

follows the theory that the higher the surface area of the cell, the greater the membrane damage owing to extracellular ice crystal formation during freezing.

Bozoglu, Ozilgen, and Bakir (1987) demonstrated an effect of cell size on the survival of LAB by determining that streptococci (small spherical cells) were more resistant to freeze drying than lactobacilli (rods). Fonseca et al. (2000) found that *Str. thermophilus* strains, being smaller in size, had greater resistance to freezing compared with *Lactobacillus* strains. Mazur (1970, 1977) proposed that cell size characteristics are related to the surface area and to the ratio of cell surface area to cell volume. When the surface area of a cell is high, membrane damage due to extracellular ice crystal formation is more extensive (Mazur, 1970). In addition, Mazur (1977) related the surface area to volume ratio of the cell and its permeability to water to the extent of intracellular freezing: the lower these factors, the greater the intracellular ice formation and resultant cell injury. This also appears to be the case for survival during storage of LAB in the freeze-dried state (Carvalho et al., 2003).

The growth medium prior to freeze drying also plays a critical role in survival subsequent to freeze drying. Compatible solutes are small molecules that can help organisms survive extreme osmotic stress (Lang, 2007). To maximize the survival of LAB postfreezing, the accumulation of compatible solutes needs to be maximized. In addition to osmoregulation, these compatible solutes help the cell to cope with the osmotic stress of the drying process during freezing (Bayles & Wilkinson, 2000). It has been demonstrated that sorbitol and glutamate have a protective effect during storage in the freeze-dried state (Carvalho et al., 2002, 2003) and it has been noted that some LAB produce limited quantities of mannitol depending on the growth medium. In most cases this mannitol remains inside the cell (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002), and thus the mannitol is probably responsible for survival during storage of freeze-dried LAB. It has also been noted that the presence of Tween 80 in the growth medium brings about changes in the fatty acid composition of LAB cells, and this is responsible for increased resistance to freezing (Goldberg & Eschar, 1977; Smitle, Gilliland, Speck, & Walter, 1974) and tolerance to bile salts (Kimoto, Ohmomo, & Okamoto, 2002).

Another important variable in the survival of LAB during freeze drying is the drying medium itself. For the dairy industry, skim milk powder is typically used as a drying medium for LAB because it stabilizes the cell membrane (Castro, Teixeira, & Kirby, 1996; Selmer-Olsen, Birkeland, & Sørhaug, 1999), creates a porous structure in the overall product that makes rehydration easier, and contains proteins that serve as a protective coating for the cells (Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001). There are three categories of protective agents that may also be added to the drying medium. The protective agent may: (1) penetrate both the cell wall and the cytoplasmic membrane (e.g., dimethyl sulfoxide and glycerol), (2) penetrate only the cell wall (e.g., oligosaccharides, amino acids, and low-molecular-weight polymers), or (3) not penetrate the cell wall or interact with the cell membrane (e.g., polymers with high molecular weight, such as proteins and polysaccharides). Hubalék (1996) has stated that the different properties of the active compounds incorporated as additives result in different protective features. For instance, compounds that are permeative act to make the cell membrane more plastic and bind water, thus reducing

dehydration and decreasing salt toxicity. Semipermeative compounds concentrate between the membrane and the cell wall, providing mechanical protection for the membrane; nonpermeative compounds form a layer on the surface of the bacteria and inhibit the rate of ice growth (Hubalék, 1996).

Storage and the methods of rehydration of freeze-dried LAB also have effects on the survival and usefulness of these cultures. Storage temperature is a critical factor, with cultures surviving best at low (−80 °C) temperatures (Abadias, Teixidó, Usall, Benabarre, & Viñas, 2001; Gardiner et al., 2000; Teixeira, Castro, & Kirby, 1995). The atmosphere in which the freeze-dried material is stored is also important. Lipids in the cell membranes of freeze-dried cultures may oxidize during storage (Castro et al., 1996; Teixeira, Castro, & Kirby, 1996); to counteract this, cultures are often stored under vacuum (Castro et al., 1996) and with controlled water activity (Teixeira, Castro, Malcata, & Kirby, 1995). Rehydration can also cause organisms to lose viability because they may have suffered sublethal injury during drying and storage and may not be able to repair the damage if they are rehydrated under inappropriate conditions (Costa, Usall, Teixidó, Garcia, & Viñas, 2000). Many factors influence the rate of recovery during the rehydration process, including the osmolarity, pH, and source of fermentable energy, as well as the temperature and volume of the rehydration solution (Poirier, Maréchal, Richard, & Gervais, 1999; Selmer-Olsen et al., 1999; Teixeira, Castro, & Kirby, 1995).

6.4.3 Effects of spray drying on LAB

Freezing and freeze drying of cultures are expensive in terms of both energy and capital equipment. Thus considerable research has been conducted on preservation by spray drying as a means of preserving starter cultures and probiotic products. The uniqueness of spray drying is that droplets are formed and dried simultaneously (Barbosa-Cánovas, Ortega-Rivas, Juliano, & Yan, 2005). During spray drying the wet product is atomized at high velocity and the spray of droplets is directed into a flow of hot air; the atomized droplets have a very large surface area resulting in a very short drying time (Morgan, Herman, White, & Vesey, 2006; Santivarangkna, Kulozik, & Foerst, 2007). Spray drying has been widely used for production of starter cultures and dehydrated probiotic bacteria (Barbosa-Cánovas & Vega-Mercado, 1996; Riveros, Ferrer, & Bórquez, 2009). Many factors affect the viability and activity of spray-dried cultures, including process and product parameters, inherent biological factors of the bacteria, and storage and rehydration conditions.

The spray drying process parameters of inlet and outlet temperature, drying time, and nozzle pressure all influence cell viability of spray-dried LAB. There are many different dryer designs and operating conditions that are selected for each dryer according to the way the product behaves during drying and the specifications needed for the end product. Peighambardoust, Golshan Taftia, and Hesaria (2011) have written an excellent review in which they detail the role of the processing parameters on spray drying LAB.

The spray drying carrier medium and concentration of solutes can also influence the viability of bacteria after spray drying. Lian, Hsiao, and Chou (2002) compared different carriers, including gelatin, gum arabic, or soluble starch, while spray drying

Bifidobacterium and determined that viability was highly dependent on the type of carriers and varied with strain. They also found that increasing the concentration of carrier from 10 to ≥20% (w/w) reduced the survival of *Bifidobacterium* (Lian et al., 2002). Typically, higher solids content of the carrier medium results in larger particle sizes that require longer drying times, subjecting entrapped bacteria to more heat damage and leading to less viability (Santivarangkna et al., 2007).

Biological parameters such as species, growth medium, growth phase, and intrinsic stress tolerance also influence the viability of spray-dried cultures. Under the same drying and storage conditions different genera and strains within a genus varied in their survival rates, and heat tolerance was not a good indicator of performance after spray drying. In one study *Lactobacillus rhamnosus* GG was the least heat resistant of the strains studied but survived the best during spray drying (Corcoran, Ross, Fitzgerald, & Stanton, 2004). Growth conditions and medium can also affect the viability of LAB during spray drying or the subsequent storage period. For instance, amino acids and sugars in the medium increase the viability of spray-dried LAB (Kets, Teunissen, & De Bont, 1996). Carvalho et al. (2004) demonstrated that supplementing growth medium with lactose yielded cells bearing the highest heat resistance. When glucose, fructose, lactose, mannose, or sorbitol was included in the drying medium the cells were better able to survive during storage (Carvalho et al., 2004). Harvesting bacterial cells at the stationary phase increases viability after spray drying (Corcoran et al., 2004; Teixeira, Castro, & Kirby, 1995; Van de Guchte et al., 2002), possibly because the stationary phase of growth provides physiological conditions that increase resistance to osmotic and heat stress as opposed to the logarithmic stage (Van de Guchte et al., 2002).

Packaging, storage conditions, and method of rehydration also affect viability and function. There is little information on the effects of packaging but one study demonstrated that spray-dried *Str. thermophilus* and *Bifidobacterium longum* survived better in laminated pouches, followed by glass bottles and polyethylene terphthalate bottles (Wang, Yu, & Chou, 2004). Additionally, a vacuum atmosphere has been demonstrated to provide a better storage environment than nitrogen or air (Chávez & Ledeboer, 2007). As is true for frozen and freeze-dried cultures, rehydration is considered to be a critical step in the recovery of spray-dried LAB. The medium used for rehydration does not appear to be critical, because in a test of four different rehydration media (at 20 °C) to revive *La. bulgaricus* there was no significant difference between skim milk; de Man, Rogosa, and Sharpe lactobacilli broth; deionized water; and phosphate buffer for rehydration (Teixeira, Castro, & Kirby, 1995; Teixeira, Castro, Malcata et al., 1995). The temperature of rehydration, however, seems to have a greater influence. A temperature increase between 4 and 50 °C was found to linearly increase the viability of *La. delbrueckii* subsp. *bulgaricus* (Teixeira, Castro, & Kirby, 1995; Teixeira, Castro, Malcata et al., 1995). Slow rehydration (30 min) led to higher cell viability than did rapid rehydration (2 min), possibly because slow soaking tends to limit the amount of osmotic shock (Teixeira, Castro, & Kirby, 1995; Teixeira, Castro, Malcata et al., 1995).

In general, spray-dried cultures compared to freeze-dried have a relatively low viability and a longer lag phase before onset of growth. Therefore, isolating new strains

of naturally thermostable LAB could be important, as well as developing encapsulation systems to improve the thermal stability of LAB. Another challenge will be to maintain the properties of LAB after spray drying and to evaluate the suitability of dried cultures in functional foods, as probiotics and biopreservatives.

6.5 Antimicrobial compounds produced by LAB: organic acids, diacetyl, and hydrogen peroxide

During lactic fermentations, the LAB produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins (Lindgren & Dobrogosz, 1990). These LAB metabolic products have a positive effect on food taste, smell, color, and texture, but they may also have the beneficial side effects of extending shelf-life and inhibiting the growth of pathogenic organisms.

6.5.1 Organic acids

End products of both homo- and hetero-LAB fermentation include organic acids such as lactic, acetic, and propionic acid, making their growth environment unfavorable for the growth of many pathogens and spoilage bacteria. Organic acids are thought to function as antimicrobials by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH, and inhibiting a variety of metabolic functions (Ricke, 2003; Ross et al., 2002). Organic acids have a very broad mode of action and inhibit both Gram-positive and Gram-negative bacteria as well as yeast and molds (Caplice & Fitzgerald, 1999). Tejero-Sarinena, Barlow, Costabile, Gibson, and Rowland (2012) investigated the antimicrobial properties of strains belonging to the *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, and *Bacillus* genera against *Salmonella* Typhimurium, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Clostridium difficile*. An agar spot test showed that most of the selected strains were able to produce active compounds antagonistic to the pathogens; cell-free culture supernatants were tested in an agar well diffusion assay and produced similar results. Neutralization of the culture supernatants with alkali reduced the antagonistic effects, indicating a probable role for organic acids in the antibacterial effects, and end product analysis demonstrated that lactic and acetic acid were the principal end products of LAB metabolism responsible for this inhibition (Tejero-Sarinena et al., 2012).

6.5.2 Diacetyl

In addition to the organic acids produced, some LAB produce diacetyl, which is generated from excess pyruvate originating from citrate (Daeschel, 1989). Diacetyl is a nonpolar, volatile diketone with a strong buttery aroma. This chemical has been reported to inhibit the growth of Gram-negative bacteria by inactivating arginine utilization through the reaction of diacetyl with the arginine-binding protein

(Jay, Rivers, & Boisvert, 1983). The antimicrobial activity of diacetyl was reviewed by Jay (1982), who screened several microbial species for sensitivity to diacetyl and concluded that this molecule has a broad antimicrobial activity at concentrations ranging between 200 and 1000 ppm, and Gram-positive bacteria were more resistant than Gram-negative bacteria. According to Jay (1982), 344 μg/ml diacetyl was inhibitory to *Listeria monocytogenes*. However, O'Bryan et al. (2009) found that diacetyl alone in amounts up to 10,000 μg/ml was not effective against *Li. monocytogenes* Scott A. Yang (2000) postulated that diacetyl may act synergistically with other antimicrobials to reduce bacterial growth, and O'Bryan et al. (2009) found that combinations of nisin and diacetyl were very effective at reducing the survival of *Li. monocytogenes* Scott A. Production of diacetyl by LAB during fermentation is typically low, approximately 4 μg/ml, so reaching inhibitory levels above 300 μg/ml would require concentration after fermentation (Earnshaw, 1992). Another practical limitation of using diacetyl as an antimicrobial is that acceptable sensory levels range from just 2−7 μg/ml, which is far below what has been shown to be an effective inhibitor (Cogan, 1980). Diacetyl used synergistically with other antimicrobials could be used at lower levels and could help contribute to the combined preservation of the food system (Yang, 2000).

6.5.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or NADH peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of enzymes and the peroxidation of membrane lipids, thus increasing membrane permeability, which can be lethal to competing organisms (Kong & Davison, 1980). The H_2O_2 may also be a precursor for the production of bactericidal free radicals such as superoxide and hydroxyl radicals, which can damage microbial DNA (Byczkowski & Gessner, 1988). It has been reported that H_2O_2 is produced in sufficient quantities by some lactobacilli to inhibit *Pseudomonas* spp., *Bacillus* spp., and *Proteus* spp. (Price & Lee, 1970), *Li. monocytogenes* (Tharrington & Sorrells, 1992), *Sta. aureus* (Dahiya & Speck, 1968), and *Sa.* Typhimurium (Watson & Schubert, 1969). Hydrogen peroxide produced by *La. delbrueckii* subsp. *bulgaricus* and *La. delbrueckii* subsp. *lactis* inhibited the growth of psychrotrophic bacteria in milk stored at 5−7 °C; no change in pH was noted (Gilliland & Ewell, 1983; Gilliland & Speck, 1975). Brashears, Reilly, and Gilliland (1998) found that *La. delbrueckii* subsp. *lactis* I produced sufficient H_2O_2 to inactivate *Es. coli* O157:H7 on refrigerated raw chicken, and compared to control samples at the end of a 5-day refrigerated storage, treated poultry breasts remained organoleptically palatable, whereas the controls were slimy with a foul odor (Brashears et al., 1998). Brashears et al. (1998) used a high level of *Lactobacillus lactis* (10^7) in their experiments with chicken; however, with the judicious selection of LAB that generate high concentrations of H_2O_2 during storage at refrigeration temperatures, there is increased potential for using LAB as an economical biopreservative at much lower levels for refrigerated foods.

6.6 Antimicrobial compounds produced by LAB: bacteriocins

The term bacteriocin is used to describe ribosomally synthesized antimicrobial compounds that are produced by many different bacterial species, including numerous members of the LAB (Jack, Tagg, & Ray, 1995). Some bacteriocins produced by LAB, such as nisin, not only inhibit closely related species but are also effective against a wide range of foodborne pathogens and many other Gram-positive spoilage microorganisms (Tagg, Dajani, & Wannamaker, 1976). Table 6.1 lists the source and activity of many bacteriocins produced by LAB. Bacteriocins, according to the classification procedure proposed by Klaenhammer (1993) and modified by Nes et al. (1996), are divided into four classes, with the majority of those produced by bacteria associated with food belonging to class I or II.

Class I bacteriocins, also known as lantibiotics, are small peptides containing the unusual amino acids lanthionine and 3-methyllanthionine, which are synthesized by Gram-positive bacteria via modification of the peptides after the protein has been translated through the endoplasmic reticulum (Stoyanova, Ustyugova, & Netrusov, 2012). Some lantibiotics have a dual mode of action. These antimicrobial peptides form a complex with the bacterial cell wall precursor lipid II, thereby inhibiting cell wall biosynthesis, after which the complex aggregates, incorporating further peptides, and forms an actual pore through the bacterial membrane (Bierbaum & Sahl, 2009). There are two types of lantibiotics, types A and B. Type A lantibiotics are linear, flexible peptides that form pores in bacterial membranes; nisin is an example of a type A lantibiotic (Mulders, Boerrigter, Rollema, Siezen, & de Vos, 1991). Type B lantibiotics are mainly globular, either uncharged or negatively charged, and inhibit the synthesis of peptidoglycan, the essential sugar−peptide backbone of the bacterial cell wall (Brötz & Sahl, 2000). Examples of type B lantibiotics are mersacidin, actagardine, and cinnamycin (Kogler et al., 1991; Schmitz, Hoffmann, Szekat, Rudd, & Bierbaum, 2006).

Class II bacteriocins include peptides with no modified amino acids and this group is divided into three subgroups, of which class IIa is the most common. Subclass IIa bacteriocins are the most thermostable, consisting of from 37−48 amino acids and possessing high activity against *Li. monocytogenes* (Drider, Fimland, Héchard, McMullen, & Prévost, 2006). Class IIa bacteriocins permeabilize the cell membrane of sensitive cells, and research has indicated that the target of these molecules is a mannose permease protein on the target cell surface (Drider et al., 2006). Subclass IIb comprises two-peptide bacteriocins and includes the α enterocins and lactococcin G peptides; they inhibit the growth of *Enterococcus* spp. and a few other Gram-positive bacteria, acting as pore-forming toxins that create cell membrane channels through a barrel-stave mechanism, thus initiating an ionic imbalance in the target cell (Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000). Subclass IIc contains circular peptides in which the N- and C-termini are covalently linked, and the circular molecule is resistant to several proteases and peptidases (Kawai, Kemperman, Kok, & Saito, 2004). These bacteriocins have various modes of action including membrane

Table 6.1 Selected bacteriocins of lactic acid bacteria (LAB)

Bacteriocin	Producing species	Bacteria inhibited	Reference
Nisin	*L. lactis* subsp *lactis*	Gram-positive	Hurst (1981)
Lacticin 3147	*L. lactis* subsp *lactis*	Gram-positive	Ryan, Rea, Hill, and Ross (1996) and McAuliffe et al. (1998)
Lacticin 481	*L. lactis* subsp *lactis*	Gram-positive	Piard, Muriana, Desmazeaud, and Klaenhammer (1992)
Lactococcin B	*L. lactis* subsp *cremoris*	Lactobacilli	Venema et al. (1993)
Enterocins A and B	*Enterococcus faecium*	*Listeria* and other Gram-positive	Casaus et al. (1997)
Lactocin 705	*L. casei*	*Listeria*, LAB, streptococci	Vignolo, Fadda, de Kairuz, de Ruiz Holgado, and Oliver (1996)
Lactacin B	*L. acidophilus*	Lactobacilli, *Lactococcus lactis*	Barefoot and Klaenhammer (1983)
Lacticin F	*L. acidophilus*	Lactobacilli, enterococci	Muriana and Klaenhammer (1991)
Sakacin	*L. sakei*	Closely related lactobacilli, *Listeria*	Schillinger and Lücke (1989)
Pediocin AcH	*P. acidilactici*	*Listeria*, LAB, other Gram-positives	Bhunia et al. (1991)
Mesentericin Y105	*Leuconostoc mesenteroides*	*Listeria*, *Leuconostoc*, lactobacilli	Héchard, Dérijard, Letellier, and Cenatiempo (1992)

permeabilization, specific inhibition of septum formation, and pheromone activity (Héchard & Sahl, 2002).

The class III and class IV bacteriocins are not well characterized. Class III bacteriocins consist of large (greater than 30 kDa) heat-labile proteins, including helveticin J produced by *Lactobacillus helveticus* (Joerger & Klaenhammer, 1986) and

enterolysin produced by *En. faecalis* (Nilsen, Nes, & Holo, 2003). The class IV bacteriocins consist of complex bacteriocins that require carbohydrate or lipid moieties for activity, but information about this class is somewhat limited and contradictory (Franz, van Belkum, Holzapfel, Abriouel, & Gálvez, 2007).

6.6.1 Nisin

Nisin is the most well known and studied of the lantibiotics. It exhibits activity against a wide array of Gram-positive bacteria including staphylococci, streptococci, bacilli, and clostridia and, even to a small degree, mycobacteria (Mota-Meira, LaPointe, Lacroix, & Lavoie, 2000). Nisin inhibits spore germination in *Bacillus* and *Clostridium* by interacting with the sulfhydryl groups in the membrane of the spore, preventing germination (Gut, Blanke, & van der Donk, 2011; Gut, Prouty, Ballard, van der Donk, & Blanke, 2008; Pol et al., 2001). In as many as 50 countries nisin has been approved for use in foods including processed and fresh cheese, canned foods, processed vegetables, and baby foods (De Vuyst & Vandamme, 1994; Hurst, 1981). Despite these endorsements, it is important to note that the refined nisin product is not currently approved as an allowable ingredient in organic food production in the United States. According to the latest Organic Materials Review Institute list, the National Organic Standards Board considers it a prohibited processing ingredient, presumably because it can, in some instances, be the product of genetically engineered bacteria (OMRI, 2013).

When a nisin-producing strain of *L. lactis* was added to the starter culture for nitrate-free Gouda cheese it prevented the outgrowth of *Clostridium tyrobutyricum* spores and also inhibited the growth of *Li. monocytogenes* in cottage and Camembert cheese (Benkerroum & Sandine, 1988). The use of bacteriocin-producing *L. lactis* subsp. *lactis* INIA 415 as a starter culture in cheese manufacturing controlled the germination of *Clostridium beijerinckii* spores and prevented late blowing defect, even under extreme conditions favorable for the germination and growth of the clostridial strain (Garde, Avila, Arias, Gaya, & Nunez, 2011). Chung, Dickson, and Crouse (1989) found that nisin delayed growth of *Li. monocytogenes* or *Sta. aureus* artificially inoculated on meats for at least 1 day at room temperature; at 5 °C, the growth of *Li. monocytogenes* was delayed for more than 2 weeks and *Sta. aureus* did not grow. However, it appears that the effectiveness of nisin applied to raw meats is limited because some meat components interfere with its activity (Henning, Metz, & Hammes, 1986). Rose, Sporns, Stiles, and McMullen (1999) used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to determine the fate of nisin in meat products stored at 4 °C overnight and concluded that nisin is apparently inactivated in raw meat by an enzymatic reaction with glutathione.

6.6.2 Pediocins

Pediocins are produced by *Pediococcus* spp. and although not very effective against spores, they are more effective than nisin in meat. Pediocin-producing cultures are readily isolated from fermented foods (Knorr, 1998; Wood, 1997). Pediocins have a narrow spectrum of activity; all pediocins are active against *Listeria* and some are

active against other Gram-positive pathogens such as *Clostridium* or *Enterococcus* (Drider et al., 2006). Eijsink, Sheie, Middelhoven, Brurberg, and Nes (1998) measured the activities of various pediocins against strains of *Li. monocytogenes* and found that pediocin AcH/PA-1 and enterocin A inhibited more strains than did sakacin P and curvacin A. It appears that pediocins target the cytoplasmic membrane of Gram-positive bacteria (Papagianni & Anastasiadou, 2009); destabilization of membranes is indicated by loss of intracellular potassium, entrance of lactose from the medium into cells, and cell lysis of some strains (Bhunia & Johnson, 1992; Bhunia, Johnson, Ray, & Kalchayanand, 1991; Motlagh et al., 1992).

6.7 Conclusions

In this chapter we have identified the long and useful role of LAB that played in preserving a broad range of foods and their pivotal role in the production of fermented foods. The genera of LAB encompass a genetically diverse group of bacteria with an equally diverse production of antimicrobial metabolites. Research on LAB has shown that a number of these natural antimicrobials produced by these microorganisms are capable of playing an expanding role in meeting the increasing consumer demand for "clean labels" on their food products. The time seems ripe for increased commercial evaluation of LAB in potential novel roles as biopreservatives and biosanitizers.

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References

Abadias, M., Benabarre, A., Teixidó, N., Usall, J., & Viñas, I. (2001). Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake*. *International Journal of Food Microbiology*, *65*, 173−182.

Abadias, M., Teixidó, N., Usall, J., Benabarre, A., & Viñas, I. (2001). Viability, efficacy, and storage of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. *Journal of Food Protection*, *64*, 856−861.

Axelsson, L. (1998). Lactic acid bacteria: classification and physiology. In S. Salminen, & A. Von Wright (Eds.), *Lactic acid bacteria: Microbiology and functional aspects* (pp. 1−72). New York: Marcel Dekker, Inc.

Balla, E., Dicks, L. M., Du Toit, M., Van Der Merwe, M. J., & Holzapfel, W. H. (2000). Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. *Applied and Environmental Microbiology*, *66*, 1298−1304.

Barbosa-Cánovas, G. V., Ortega-Rivas, E., Juliano, P., & Yan, H. (2005). *Food powders: Physical properties, processing, and functionality*. New York: Kluwer Academic/Plenum Publishers.

Barbosa-Cánovas, G. V., & Vega-Mercado, H. (1996). *Dehydration of foods*. New York: Chapman & Hall.

Barefoot, S. F., & Klaenhammer, T. R. (1983). Detection and activity of lactacin-B, a bacteriocin produced by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*, *45*, 1808−1815.

Bayles, D. O., & Wilkinson, B. J. (2000). Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. *Letters in Applied Microbiology*, *30*, 23−27.

Benkerroum, N., & Sandine, W. E. (1988). Inhibitory action of nisin against *Listeria monocytogenes*. *Journal of Dairy Science*, *71*, 3237−3245.

Bhunia, A. K., & Johnson, M. G. (1992). Monoclonal antibody-colony immunoblot method specific for isolation of *Pediococcus acidilactici* from foods and correlation with pediocin (bacteriocin) production. *Applied and Environmental Microbiology*, *58*, 2315−2320.

Bhunia, A. K., Johnson, M. C., Ray, B., & Kalchayanand, N. (1991). Mode of action of pediocin ACH from *Pediococcus acidilactici* H on sensitive bacterial strains. *Journal of Applied Bacteriology*, *70*, 25−33.

Bierbaum, G., & Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Current Pharmaceutical Biotechnology*, *10*, 2−18.

Bozoglu, T. F., Ozilgen, M., & Bakir, U. (1987). Survival kinetics of lactic acid starter cultures during and after freeze drying. *Enzyme and Microbial Technology*, *9*, 531−537.

Brashears, M. M., Reilly, S. S., & Gilliland, S. E. (1998). Antagonistic action of cells of *Lactobacillus lactis* toward *Escherichia coli* O157:H7 on refrigerated raw chicken meat. *Journal of Food Protection*, *61*, 166−170.

Broadbent, J. R., & Lin, C. (1999). Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology*, *39*, 88−102.

Brötz, H., & Sahl, H.-G. (2000). New insights into the mechanism of action of lantibiotics − diverse biological effects by binding to the same molecular target. *Journal of Antimicrobial Chemotherapy*, *46*, 1−6.

Byczkowski, J. Z., & Gessner, T. (1988). Biological role of superoxide ion-radical. *International Journal of Biochemistry*, *20*, 569−580.

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*, 131−149.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2002). Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protectants. *Biotechnology Letters*, *24*, 1587−1591.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2003). Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. *Le Lait*, *83*, 203−210.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2004). Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp *bulgaricus*. *Biotechnology Progress*, *20*, 248−254.

Casaus, P., Nilsen, T., Cintas, L. M., Nes, I. F., Hernández, P. E., & Holo, H. (1997). Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. *Microbiology*, *143*, 2287−2294.

Castro, H. P., Teixeira, P. M., & Kirby, R. (1996). Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. *Biotechnology Letters*, *18*, 99−104.

Chávez, B. E., & Ledeboer, A. M. (2007). Drying of probiotics: optimization of formulation and process to enhance storage survival. *Drying Technology*, *25*, 1193−1201.

Chung, K.-T., Dickson, J. S., & Crouse, J. D. (1989). Effects of nisin on growth of bacteria attached to meat. *Applied and Environmental Microbiology*, *55*, 1329−1333.

Cogan, T. M. (1980). Les levains lactiques mésophiles: Une revue. *Le Lait*, *60*, 397−425.

Corcoran, B. M., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. *Journal of Applied Microbiology*, *96*, 1024−1039.

Costa, E., Usall, J., Teixidó, N., Garcia, N., & Viñas, I. (2000). Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology*, *89*, 793−800.

Daeschel, A. M. (1989). Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technology*, *43*, 164−167.

Dahiya, R. S., & Speck, M. L. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *Journal of Dairy Science*, *51*, 1568−1572.

De Vuyst, L., & Vandamme, E. J. (1994). Lactic acid bacteria and bacteriocins: their practical importance. In L. De Vuyst, & E. J. Vandamme (Eds.), *Bacteriocins of lactic acid bacteria, microbiology, genetics and application* (pp. 1−12). New York: Springer Science+ Business Media, LLC.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, *70*, 564−582.

Dunn, M. S., Shankman, S., Camien, M. N., & Block, H. (1947). The amino acid requirements of twenty-three lactic acid bacteria. *Journal of Biological Chemistry*, *168*, 1−22.

Earnshaw, R. G. (1992). The antimicrobial action of lactic acid bacteria: natural food preservation systems. In B. J. B. Wood (Ed.), *The lactic acid bacteria in health and disease* (pp. 211−232). London and New York: Elsevier Applied Science.

Eijsink, V. G., Sheie, M., Middelhoven, P. H., Brurberg, M. B., & Nes, I. F. (1998). Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Applied and Environmental Microbiology*, *64*, 3275−3281.

Fernández, M., & Zúñiga, M. (2006). Amino acid catabolic pathways of lactic acid bacteria. *Critical Reviews in Microbiology*, *32*, 155−183.

Fonseca, F., Béal, C., & Corrieu, G. (2000). Method for quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. *Journal of Dairy Research*, *67*, 83−90.

Fonseca, F., Béal, C., & Corrieu, G. (2001). Operating conditions that affect the resistance of lactic acid bacteria to freezing and frozen storage. *Cryobiology*, *43*, 189−198.

Fonseca, F., Marin, M., & Morris, G. J. (2006). Stabilization of frozen *Lactobacillus delbrueckii* subsp. *bulgaricus* in glycerol suspensions: freezing kinetics and storage temperature effects. *Applied and Environmental Microbiology*, *72*, 6474−6482.

Fox, P. F. (1993). Cheese: an overview. In P. F. Fox (Ed.), *Cheese: Chemistry, physics and microbiology* (pp. 1−36). London: Chapman and Hall.

Franz, C. M. A. P., van Belkum, M. J., Holzapfel, W. H., Abriouel, H., & Gálvez, A. (2007). Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews*, *31*, 293−310.

Garde, S., Avila, M., Arias, R., Gaya, P., & Nunez, M. (2011). Outgrowth inhibition of *Clostridium beijerinckii* spores by a bacteriocin-producing lactic culture in ovine milk cheese. *International Journal of Food Microbiology*, *150*, 59−65.

Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M. A. E., Fitzgerald, G. F., Collins, J. K., et al. (2000). Comparative survival rates of human-derived probiotic *Lactobacillus paracasei*

and *L. salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology*, *66*, 2605−2612.
Gilliland, S. E., & Ewell, H. R. (1983). Influence of combination of *Lactobacillus lactis* and potassium sorbate on growth of psychrotrophs in raw milk. *Journal of Dairy Science*, *66*, 974−980.
Gilliland, S. E., & Speck, M. L. (1975). Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. *Journal of Food Science*, *40*, 903−905.
Goldberg, I., & Eschar, L. (1977). Stability of lactic acid bacteria to freezing as related to their fatty acid composition. *Journal of Dairy Science*, *33*, 489−496.
Gut, I. M., Blanke, S. R., & van der Donk, W. A. (2011). Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. *ACS Chemical Biology*, *6*, 744−752.
Gut, I. M., Prouty, A. M., Ballard, J. D., van der Donk, W. A., & Blanke, S. R. (2008). Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrobial Agents and Chemotherapy*, *52*, 4281−4288.
Hansen, E. B. (2002). Commercial bacterial starter cultures for fermented foods of the future. *International Journal of Food Microbiology*, *78*, 119−131.
Héchard, Y., Dérijard, B., Letellier, F., & Cenatiempo, Y. (1992). Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. *Journal of General Microbiology*, *138*, 2725−2731.
Héchard, Y., & Sahl, H. G. (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie*, *84*, 545−557.
Henning, S., Metz, R., & Hammes, W. P. (1986). Studies on the mode of action of nisin. *International Journal of Food Microbiology*, *3*, 121−134.
Hubalék, Z. (1996). *Cryopreservation of microorganisms at ultra-low temperatures*. Czech Republic: Academia Praha.
Hurst, A. (1981). Nisin. *Advances in Applied Microbiology*, *27*, 85−123.
Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of Gram-positive bacteria. *Microbiological Reviews*, *59*, 171−200.
Jay, J. M. (1982). Antimicrobial properties of diacetyl. *Applied and Environmental Microbiology*, *44*, 525−532.
Jay, J. M., Rivers, G. M., & Boisvert, W. E. (1983). Antimicrobial properties of α-dicarbonyl and related compounds. *Journal of Food Protection*, *46*, 325−329.
Joerger, M. C., & Klaenhammer, T. R. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *Journal of Bacteriology*, *167*, 439−446.
Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek Journal of Microbiology*, *49*, 209−224.
Karlsson, J. O. M., Cravalho, E. G., & Toner, M. (1993). Intracellular ice formation: causes and consequences. *Cryo Letters*, *14*, 323−334.
Kawai, Y., Kemperman, R., Kok, J., & Saito, T. (2004). The circular bacteriocins gassericin A and circularin A. *Current Protein & Peptide Science*, *5*, 393−398.
Kets, E. P. W., Teunissen, P. J. M., & De Bont, J. A. M. (1996). Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Applied and Environmental Microbiology*, *62*, 259−261.
Kimoto, H., Ohmomo, S., & Okamoto, T. (2002). Enhancement of bile tolerance in lactococci by Tween 80. *Journal of Applied Microbiology*, *92*, 41−46.
Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, *12*, 39−85.

Knorr, D. (1998). Technological aspects related to microorganisms in functional foods. *Trends in Food Science and Technology*, *9*, 295−306.

Kogler, H., Bauch, M., Fehlhaber, H.-W., Griesinger, C., Schubert, W., & Teetz, V. (1991). NMR-spectroscopic investigations on mersacidin. In G. Jung, & H.-G. Sahl (Eds.), *Nisin and novel lantibiotics* (pp. 159−170). Leiden: ESCOM.

König, H., & Fröhlich, J. (2009). Lactic acid bacteria. In H. König, G. Unden, & J. Fröhlich (Eds.), *Biology of microorganisms on grapes, in must and in wine* (pp. 3−30). Berlin: Springer-Verlag.

Kong, S., & Davison, A. J. (1980). The role of interactions between O_2, H_2O_2, $\cdot OH$, e^- and O_2^- in free radical damage to biological systems. *Archives of Biochemistry and Biophysics*, *204*, 18−29.

Lang, F. (2007). Mechanisms and significance of cell volume regulation. *Journal of the American College of Nutrition*, *26*, 613S−623S.

Lian, W. C., Hsiao, H. C., & Chou, C. C. (2002). Survival of bifidobacteria after spray-drying. *International Journal of Food Microbiology*, *74*, 79−86.

Lindgren, S. E., & Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews*, *7*, 149−163.

Mazur, P. (1966). Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing. In H. T. Meryman (Ed.), *Cryobiology* (pp. 213−315). London, UK: Academic Press.

Mazur, P. (1970). Cryobiology: the freezing of biological systems. *Science*, *168*, 939−949.

Mazur, P. (1977). The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology*, *14*, 251−272.

McAuliffe, O., Ryan, M. P., Ross, R. P., Hill, C., Breeuwer, P., & Abee, T. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Applied and Environmental Microbiology*, *64*, 439−445.

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying: a review. *Journal of Microbiological Methods*, *66*, 183−193.

Morichi, T., Irie, R., Yano, N., & Kembo, H. (1963). Protective effect of glutamic acid and related compounds on bacterial cells subjected to freeze-drying. *Journal of General and Applied Microbiology*, *9*, 149−161.

Mota-Meira, M., LaPointe, G., Lacroix, C., & Lavoie, M. C. (2000). MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. *Antimicrobial Agents and Chemotherapy*, *44*, 24−29.

Motlagh, A. M., Bhunia, A. K., Szostek, F., Hansen, T. R., Johnson, M. C., & Ray, B. (1992). Nucleotide and amino acid sequence of *pap*-gene (pediocin AcH production) in *Pediococcus acidilactici H*. *Letters in Applied Microbiology*, *15*, 45−48.

Mulders, J. W. M., Boerrigter, I. J., Rollema, H. S., Siezen, R. J., & de Vos, W. M. (1991). Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *European Journal of Biochemistry*, *201*, 581−584.

Muriana, P. M., & Klaenhammer, T. R. (1991). Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Applied and Environmental Microbiology*, *57*, 114−121.

Nes, I. F., Diep, D. B., Håvarstein, L. S., Brurberg, M. B., Eijsink, V., & Holo, H. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek Journal of General and Molecular Microbiology*, *70*, 113−128.

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Applied and Environmental Microbiology*, *69*, 2975−2984.

O'Bryan, C. A., Sostrin, M. L., Nannapaneni, R., Ricke, S. C., Crandall, P. G., & Johnson, M. G. (2009). Sensitivity of *Listeria monocytogenes* Scott A to nisin and diacetyl after starvation in sodium phosphate buffered saline. *Journal of Food Science*, *74*, M493−M498.

OMRI. (2013). *OMRI products list*. http://www.omri.org/omri-lists/download.

Papagianni, M., & Anastasiadou, S. (2009). Pediocins: the bacteriocins of Pediococci Sources, production, properties and applications. *Microbial Cell Factories*, *8*. Article Number: 3. DOI: 10.1186/1475-2859-8-3.

Pederson, C. S. (1979). *Microbiology of food fermentations* (2nd ed.). Westport, CT: Avi Publishing Co.

Peighambardousta, S. H., Golshan Taftia, A., & Hesaria, J. (2011). Application of spray drying for preservation of lactic acid starter cultures: a review. *Trends in Food Science and Technology*, *22*, 215−224.

Piard, J. C., Muriana, P. M., Desmazeaud, M. J., & Klaenhammer, T. R. (1992). Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481. *Applied and Environmental Microbiology*, *58*, 279−284.

Picque, D., Perret, B., Latrille, E., & Corrieu, G. (1992). Characterization and classification of lactic acid bacteria based on their acidification kinetics. *Lebensmittel-Wissenschaft und -Technologie*, *25*, 181−186.

Poirier, I., Maréchal, P.-A., Richard, S., & Gervais, P. (1999). *Saccharomyces cerevisiae* viability is strongly dependent on rehydration kinetics and the temperature of dried cells. *Journal of Applied Microbiology*, *86*, 87−92.

Pol, I. E., van Arendonk, W. G. C., Mastwijk, H. C., Krommer, J., Smid, E. J., & Moezelaar, R. (2001). Sensitivities of germinating spores and carvacrol-adapted vegetative cells and spores of *Bacillus cereus* to nisin and pulsed-electric-field treatment. *Applied and Environmental Microbiology*, *67*, 1693−1699.

Price, R. J., & Lee, J. S. (1970). Inhibition of *Pseudomonas* spp. by hydrogen peroxide producing lactobacilli. *Journal of Milk and Food Technology*, *33*, 13−16.

Ray, B., & Daeschel, M. (1992). *Food biopreservatives of microbial origin*. Boca Raton, FL: CRC Press.

Reddy, G., Altaf, M., Naveena, B. J., Venkateshwar, M., & Vijay Kumar, E. (2008). Amylolytic bacterial lactic acid fermentation − a review. *Biotechnolology Advances*, *26*, 22−34.

Ricke, S. C. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science*, *82*, 632−639.

Ricke, S. C., Koo, O. K., & Keeton, J. T. (2013). Fermented meat, poultry, and fish products. In M. P. Doyle, & R. L. Buchanan (Eds.), *Food microbiology: Fundamentals and frontiers* (4th ed.). (pp. 857−880). Washington, DC: American Society for Microbiology.

Riveros, B., Ferrer, J., & Bórquez, R. (2009). Spray drying of a vaginal probiotic strain of *Lactobacillus acidophilus*. *Drying Technology*, *27*, 123−132.

Rose, N. L., Sporns, P., Stiles, M. E., & McMullen, L. M. (1999). Inactivation of nisin by glutathione in fresh meat. *Journal of Food Science*, *64*, 759−762.

Ross, R. P., Morgan, S., & Hill, C. (2002). Preservation and fermentation: past, present and future. *International Journal of Food Microbiology*, *79*, 3−16.

Ryan, M. P., Rea, M. C., Hill, C., & Ross, R. P. (1996). An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Applied and Environmental Microbiology*, *62*, 612−619.

Santivarangkna, C., Kulozik, U., & Foerst, P. (2007). Alternative drying processes for the industrial preservation of lactic acid starter cultures. *Biotechnology Progress*, *23*, 302−315.

Schillinger, U., & Lücke, F.-K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology*, *55*, 1901−1906.

Schmitz, S., Hoffmann, A., Szekat, C., Rudd, B., & Bierbaum, G. (2006). The lantibiotic mersacidin is an autoinducing peptide. *Applied and Environmental Microbiology*, *72*, 7270−7277.

Selmer-Olsen, E., Birkeland, S.-E., & Sørhaug, T. (1999). Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. *Journal of Applied Microbiology*, *87*, 429−437.

Smitle, R. B., Gilliland, S. E., Speck, M. L., & Walter, W. M. (1974). Relationship of cellular fatty acid composition to survival of *Lactobacillus bulgaricus* in liquid nitrogen. *Applied Microbiology*, *27*, 738−743.

Stoyanova, L. G., Ustyugova, E. A., & Netrusov, A. I. (2012). Antibacterial metabolites of lactic acid bacteria: their diversity and properties. *Applied Biochemistry and Microbiology*, *48*, 229−243.

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, *36*, 1−29.

Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriology Reviews*, *40*, 722−756.

Teixeira, P., Castro, H., & Kirby, R. (1995). Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. *Journal of Applied Microbiology*, *78*, 456−462.

Teixeira, P. C., Castro, M. H., Malcata, F. X., & Kirby, R. M. (1995). Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* following spray-drying. *Journal of Dairy Science*, *78*, 1025−1031.

Teixeira, P., Castro, H., & Kirby, R. (1996). Evidence of membrane lipid oxidation of spray-dried *Lactobacillus bulgaricus* during storage. *Letters in Applied Microbiology*, *22*, 34−38.

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G. R., & Rowland, I. (2012). *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: evidence for the effects of organic acids. *Anaerobe*, *18*, 530−538.

Tharrington, G., & Sorrells, K. M. (1992). Inhibition of *Listeria monocytogenes* by milk culture filtrates from *Lactobacillus delbrueckii* subsp. *Lactis*. *Journal of Food Protection*, *55*, 542−544.

Tsvetkov, T., & Shishkova, I. (1982). Studies on the effects of low temperatures on lactic acid bacteria. *Cryobiology*, *19*, 211−214.

Van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S. D., & Maguin, E. (2002). Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek Journal of General and Molecular Microbiology*, *82*, 187−216.

Vandamme, P., Pot, B., Gilllis, M., de Vos, P., Kersters, K., & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Reviews*, *60*, 407−438.

Venema, K., Abee, T., Haandrikman, A. J., Leenhouts, K. J., Kok, J., Konings, W. N., et al. (1993). Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. *Applied and Environmental Microbiology*, *59*, 1041−1048.

Vignolo, G., Fadda, S., de Kairuz, M. N., de Ruiz Holgado, A. A., & Oliver, G. (1996). Control of *Listeria monocytogenes* in ground beef by 'Lactocin 705', a bacteriocin produced by *Lactobacillus casei* CRL 705. *International Journal of Food Microbiology*, *29*, 397−402.

Wang, Y.-C., Yu, R.-C., & Chou, C.-C. (2004). Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. *International Journal of Food Microbiology*, *93*, 209−217.

Watson, J. A., & Schubert, J. (1969). Action of hydrogen peroxide on growth inhibition of *Salmonella typhimurium*. *Journal of General Microbiology*, *57*, 25−34.

Wibowo, D., Eschenbruch, R., Davis, C. R., Fleet, G. H., & Lee, T. H. (1985). Occurrence and growth of lactic acid bacteria in wine: a review. *American Journal of Enology and Viticulture*, *36*, 302−313.

Wisselink, H. W., Weusthuis, R. A., Eggink, G., Hugenholtz, J., & Grobben, G. J. (2002). Mannitol production by lactic acid bacteria: a review. *International Dairy Journal*, *12*, 151−161.

Wood, B. J. B. (1997). *Microbiology of fermented foods* (2nd ed.). New York, NY: Blackie A&P.

Yang, Z. (2000). *Antimicrobial compounds and extracellular polysaccharides produced by lactic acid bacteria: Structures and properties* (Ph.D. dissertation). Department of Food Technology, University of Helsinki.

Lactic acid bacteria (LAB) as antimicrobials in food products: analytical methods and applications

7

C.A. O'Bryan[1], P.G. Crandall[1], S.C. Ricke[1], J.B. Ndahetuye[2]
[1]University of Arkansas, Fayetteville, AR, USA; [2]Institute of Agriculture and Animal Husbandry, Kigali, Africa

7.1 Introduction

This chapter builds on the previous chapter, which reviewed the main types and characteristics of LAB. The chapter reviews key issues in using LAB in practice for food preservation. It first summarizes key methods for screening LAB for antimicrobial activity. The chapter then outlines the regulatory framework governing the use of LAB in foods. It then describes the main methods for using LAB as biopreservatives in food. It concludes by reviewing the use of LAB in the preservation of particular foods and as a biosanitizer.

7.2 Screening lactic acid bacteria (LAB) for antimicrobial activity

Lactic acid bacteria (LAB) isolated from different sources such as plant material, food products, or human and animals can be screened for antimicrobial activity, and it is very important to be able to screen a large number of strains in an easy, rapid, and reliable way. There are several screening methods available to detect antimicrobial activity from LAB strains. The following methods will be discussed in more detail in this chapter:

- Spot-on-lawn assay
- Disc diffusion
- Microtiter plate assay
- Agar well diffusion assay
- Multiwell plate assay

One parameter to consider when conducting screening assays is the particular culture medium being used to grow the sensitive strain. Recent experiments indicate that, when a sensitive strain is grown in a medium that contains enzymatic casein hydrolysate, smaller zones of inhibition are formed than when grown in a medium with

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acid hydrolysate of casein (Salvucci, Saavedra, & Sesma, 2007). The presence of more medium-sized peptides in the enzymatic hydrolysate may interfere in some way with the bacteriocin activity, perhaps by the formation of protein aggregates (Vera Pingitore, Hebert, Sesma, & Nader-Macías, 2009).

Moraes et al. (2010) evaluated culture media and methods of isolation and detection of LAB producing bacteriocin-like substances using *Listeria monocytogenes* ATCC 7644 as an indicator. They compared de Man−Rogosa−Sharpe (MRS) agar and Kang−Fung−Sol agar (KFS), and the use of the spot-on-the-lawn and well-diffusion assays. A larger number of antagonist cultures were isolated on MRS (148) than on KFS (39), and the spot-on-the-lawn assay identified a higher frequency of LAB capable of producing bacteriocin-like substances. The most frequently used screening methods, as well as some innovative methods, will be described in this section.

7.2.1 Spot-on-lawn assay

The simplest direct test and the one most widely used for the preliminary screening of large numbers of LAB strains is the spot-on-lawn assay, based on the method devised by Gratia (1946). Here, the test and indicator cultures are grown simultaneously, and the demonstration of antagonism is dependent on the release of a diffusible inhibitor early in the growth of the test culture. In the simplest form of this test, the sensitive organism is inoculated onto the surface of the appropriate solid medium in a petri dish and allowed to dry. Subsequently, drops of test cultures are placed on different segments of the plate, and the plates are incubated and observed for zones of clearing around the drops (Kuttner, 1966). In the most commonly used modification, the indicator (sensitive) bacterium is inoculated into tempered (50 °C) soft agar (0.7% agar), which is poured onto the surface of prepared agar plates of the appropriate medium and allowed to solidify. Microliter aliquots of overnight cultures or cell-free supernatants (CFS) of cultures are spotted onto this lawn and plates incubated in an upright position; zones of inhibition are subsequently measured after an overnight incubation (Braun, Schaller, & Wabl, 1974; Milillo et al., 2013).

7.2.2 Disc diffusion assay

The disc diffusion method was originally developed as a means of measuring the effect of an antibiotic against bacteria grown in culture (Bauer, Kirby, Sherris, & Turck, 1966). The bacterium in question is swabbed uniformly across a culture plate. A 6-mm filter-paper disc, impregnated with a known dilution of the antimicrobial compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disc, and will decrease as distance from the disc increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This creates a measurable zone of inhibition. Thus, the size of the zone of inhibition is a measure of the

effectiveness of the compound: the larger the clear area around the filter disc, the more effective the compound at lower concentrations against the test bacterial species.

The disc diffusion method has been adapted for use in testing cultures or compounds for antimicrobial action by placing an aliquot of culture or compound onto 6-mm diameter sterile discs, which are allowed to dry for 10 min. The sensitive organism is spread, plated onto the appropriate medium and the discs are aseptically placed on the surface of the agar; plates are incubated overnight and the zones of inhibition measured (Meena & Sethi, 1994). Tadese, Ephraim, and Ashenafi (2005) modified this method somewhat by dipping sterile filter discs into the culture broth of LAB that had been incubated for 24 h. These discs were placed on solidified Mueller−Hinton agar seeded with 12−14 h cultures of test organisms; plates were incubated overnight and zones of inhibition measured.

7.2.3 Agar well diffusion assay

A horizontal agar diffusion method to assay nisin was developed by Tramer and Fowler (1964) utilizing *Micrococcus luteus* as the test organism. Molten agar was tempered to 40 °C and inoculated with the indicator organism at the middle of exponential phase to a final concentration of 1%. The inoculated medium was poured in sterile petri dishes and allowed to solidify. After agar solidification, wells of 5-mm diameter were cut in the solid agar with the use of a glass tube. Rogers and Montville (1991) increased the sensitivity of this method by using *Lactobacillus sakei* ATCC 15,521 as the indicator organism for nisin assays. Tagg and McGiven (1971) developed a refinement of this method to study other organisms and potential antimicrobials. They filled petri dishes with an appropriate nutrient medium to a thickness of 5 mm, and used a sterile 4-mm cork borer to punch holes out of the agar. The base of each hole was sealed with 0.05 ml of melted nutrient agar, and standardized quantities of inhibitory bacterial culture test preparations were subsequently added to the appropriate wells. These plates were incubated for 1−2 h to allow for the diffusion of the antimicrobial metabolites into the medium. The agar was then removed from the petri dish with a sterile spatula, inverted into the lid of the petri dish, exposing the bottom surface of the agar followed by flooding with a log phase culture of the appropriate indicator organism. The plates were drained, dried, and incubated with the lids uppermost until zones of inhibition clearly developed.

Ham et al. (2003) modified this method by placing metal borers (8 mm) in the petri dish prior to pouring medium inoculated with the sensitive strain into the dish. The borers were removed after the medium solidified and the base of each hole was sealed with 0.05 ml of melted agar. Aliquots of test culture were added to the wells, and plates incubated for at least a day before examining for zones of inhibition. Mallesha, Selvakumar, and Jagannath (2010) further refined the method by inoculating 0.1 ml of 18 h cultures of sensitive bacteria onto previously poured and set nutrient agar plates by the spread plate method. Subsequently, four wells of 8-mm diameter were aseptically made in each of the plates, and filled with 100 µl of test compound. Plates were incubated overnight before measuring zones of inhibition.

7.2.4 Microtiter plate assays

Kang and Fung (1998) developed a microtiter plate assay for screening LAB for activity against *Li. monocytogenes*. Individual colonies of LAB were transferred with sterile toothpicks into individual wells of a microtiter plate containing 50 µl BHI broth per well and the plates were incubated in an anaerobic atmosphere for 24 h. After incubation, 50 µl of modified Oxford medium (MOX) containing *Li. monocytogenes* (5.0 $\log_{10}$ CFU/ml) was added to each well of the microtiter plate; LAB do not grow in MOX medium, whereas *Li. monocytogenes* grows and produces a characteristic black color. If the LAB do not inhibit the *Li. monocytogenes*, the medium will turn black; if the LAB are inhibitory, no growth occurs and the medium remains clear.

7.2.5 Multiwell plate assays

Yaakoubi et al. (2009) modified the method of Kang and Fung (1998) to develop what they termed the multiwell antagonistic activity assay. The LAB to be tested for antagonistic activity was grown in MRS broth overnight and a fraction of this culture centrifuged to produce a CFS. Since these researchers were interested only in bacteriocins, they neutralized the CFS with NaOH and treated it with catalase to eliminate the effects of organic acids and H_2O_2, respectively. A 130 µl aliquot of the CFS was dispensed into individual wells of a microtiter plate containing 750 µl of broth inoculated with 10 µL of the sensitive organism. The plate was subsequently held at 15 °C, and the optical density (600 nm) was determined at regular intervals using a spectrophotometer.

7.2.6 Innovative methods of screening for bacteriocins

A genome mining software tool, BAGEL2 (de Jong, van Heel, Kok, & Kuipers, 2010), was used by Virolainen, Guglielmetti, Arioli, and Karp (2012) to identify seven presumed lantibiotic genes within three bacterial genomes. However, the presence of these genes does not mean they will be expressed, as several studies have reported the presence of nisin genes in strains that do not produce nisin (Moschetti, Villani, Blaiotta, Baldinelli, & Coppola, 1996; de Vuyst, 1994). For this reason, Virolainen et al. (2012) developed an application of the bioluminescent whole-cell nisin biosensor *Lactobacillus lactis* subsp. *cremoris* NZ9800lux (Immonen & Karp, 2007) for screening nisin producers. An overlay of the biosensor strain on microbial colonies was presumed to produce nisin yields identification results within 1 h. They tested the method by screening nisin producers among 144 colonies obtained from raw milk and a panel of 91 lactococci. Only colonies later verified as nisin producers induced bioluminescence in the biosensor strain, and none of the 11 strains that produced known bacteriocins other than nisin or uncharacterized bacteriocins induced bioluminescence.

It may be important to test by more than one basic method when screening strains for inhibitory activity, and to use a wide range of media and growth conditions. The optimal conditions for growth of the test strain may not be the ideal conditions for

production of bacteriocins or other antimicrobial compounds. The composition of the medium may also play a role in affecting how sensitive an indicator strain is under these growth conditions. When some strains of *Streptococcus mutans* and *Streptococcus salivarius* are grown on sucrose-supplemented medium, these normally susceptible organisms are resistant to a bacteriocin produced by *St. mutans* (Rogers, 1974). Application of basic screening procedures provides useful preliminary information for the identification of LAB with antimicrobial effects on pathogens. As always, it is vital to remember that these are screening methods, and follow-on confirmation is required using a matrix that includes the actual food to be protected.

7.3 Regulatory framework governing the use of LAB in food

The group of LAB is central to the production of fermented foods and beverages, and these organisms have a long and safe history of food applications and human consumption (Lindgren & Dobrogosz, 1990). These bacteria may be present as natural flora of the food, or as a result of intentional addition as starter cultures in an industrial food fermentation process. Additionally, the metabolites of LAB have been consumed in large quantities by countless generations of people in cultured foods with no adverse effects, so LAB are a preferred source for food-use bacteriocins, either in the form of purified compounds or growth extracts.

7.3.1 Regulatory environment of the United States

In the United States, food and food additives are regulated by the Food, Drug, and Cosmetic Act of 1938 (U.S. Food and Drug Administration, 2011). This act was amended in 1958 to include a list of substances that were exempt from the then new requirement that manufacturers test food additives before putting them on the market; this introduced the concept of "generally recognized as safe" (GRAS) (U.S. Food and Drug Administration, 2013). A GRAS substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use. A substance recognized for such use prior to 1958 is by default GRAS. Since LAB have been part of fermented foods for centuries, LAB in these products can be said to be GRAS by default. However, because they are GRAS for a specific food use, they are not necessarily GRAS for all food uses. It is the use of a substance rather than the substance itself that is GRAS, as the safety determination is always limited to its intended conditions of usage. When microorganisms with a safe history in food are employed for a different use or at a significantly higher dosage, a GRAS determination must be obtained. A person or company can "self affirm" by informing the U.S. Food & Drug Administration (FDA) of a determination that the use of a substance is GRAS based on scientific procedures or prior sanction; the FDA may at that point issue a letter of no objection for this defined use.

One GRAS notice has been submitted to the FDA related to the use of live microorganisms as biopreservatives: GRN 000,171 states that the combination of *Lactobacillus acidophilus*, *La. lactis*, and *Pediococcus acidilactici* are GRAS for use only in meat and poultry products to control pathogens. A second GRAS notice, GRN 000,159, has been submitted for GRAS status for an antilisterial food antimicrobial composed of *Carnobacterium maltaromaticum*, which is considered by many researchers to be a member of the LAB group, although it is genetically distinct from that group.

7.3.2 Regulatory environment in the European Union

The European Food Safety Authority (EFSA) has recommended that panels use a common risk assessment approach to ensure consistency in granting approvals for the use of antimicrobials: the Qualified Presumption of Safety (QPS). The QPS approach is meant to be a fast track for species for which there is a sufficient body of knowledge that all strains within the species are assumed to be safe. This presumption may be qualified by some restrictions, such as the absence of specific characteristics (for example, the absence of transmissible antibiotic resistance, absence of food poisoning toxins, absence of surfactant activity, and absence of enterotoxic activity). The QPS list covers only selected groups of microorganisms that have been referred to EFSA for a formal assessment of safety (Leuschner et al., 2010). The list is updated annually (EFSA, 2013). The absence of a particular organism from the QPS list does not necessarily imply a risk associated with its use. Individual strains may be safe, but this cannot be ascertained from the existing knowledge of the taxonomic unit to which it belongs. Another reason that a species is not listed could be that EFSA has not been asked to assess the safety of any strains of the species. A recent review (Herody, Soyeux, Hansen, & Gillies, 2010) gives a thorough description of the European regulatory environment for microbial food cultures. The reader is referred to the most recent QPS lists for information on specific genera and species of LAB on the list (EFSA, 2013).

7.4 Methods for using LAB as biopreservatives in food

The use of nonpathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods is termed *biopreservation* (Stiles, 1996). Several means of using LAB as biopreservatives have been studied, including:

- using LAB with known antimicrobial activity
- using a previously fermented product in a formulation or as a starter culture
- using purified bacteriocins produced by LAB

This section reviews the two most commonly used methods: the use of LAB with known antimicrobial activity and the use of bacteriocins produced by LAB.

7.4.1 Use of LAB with known antimicrobial activity

Brashears, Reilly, and Gilliland (1998) reported that a selected strain of *La. lactis* (*Lactobacillus delbreuckii* subsp. *lactis*) inhibited *Escherichia coli* O157:H7 on raw

chicken meat during refrigerated storage. These scientists also reported that the lactobacilli were able to exert antagonistic action toward coliforms and psychrotrophic spoilage flora. Several species of *Salmonella*, associated with food products including meats, fruits, and vegetables, were inhibited by *La. lactis* (*La. delbreuckii* subsp. *lactis*) during incubation at 37 °C (Brashears & Durre, 1999).

Hugas, Pagés, Garriga, and Monfort (1998) studied the effects of adding *La. sakei* CTC494 to raw minced pork, poultry breasts, and cooked pork on the growth of *Listeria* stored under different atmospheres; they achieved the greatest inhibition of *Listeria* in vacuum-packaged samples of poultry breasts and cooked pork, and in the modified-atmosphere packaged samples of raw minced pork. Bredholt, Nesbakken, and Holck (1999) investigated the use of LAB found naturally in cooked meat products as protective cultures. They inoculated cooked meat products with *Li. monocytogenes* and studied the growth of the *Li. monocytogenes* and native LAB during 4 weeks of storage at refrigeration temperatures. They isolated the LAB from products in which *Li. monocytogenes* failed to grow, and selected five different strains for *in vitro* testing against *Li. monocytogenes* and *E. coli* O157:H7, and found that all five strains inhibited growth of both pathogens. A professional taste panel evaluated cooked, sliced, and vacuum-packaged ham inoculated with each of the five test strains after storage for 21 days at 8 °C, and found that all samples had acceptable sensory properties. They subsequently used the cultures, all identified as *La. sakei*, in cooked meat products, and achieved inhibition of *Li. monocytogenes* growth both at 8 °C and 4 °C (Bredholt, Nesbakken, & Holck, 2001). Consumer acceptance tests of cooked ham and sausage found that control and inoculated products were equally acceptable. Koo, Eggleton, O'Bryan, Crandall, and Ricke (2012) found that a combination of three LAB strains (LactiGuard™) inhibited the growth of *Li. monocytogenes* inoculated onto frankfurters not containing lactate/diacetate by nearly 1.0 $\log_{10}$ during 8 weeks of refrigerated storage.

7.4.2 Use of bacteriocins in food

Nisin was the first bacteriocin approved for use in food. It is commercially produced using a culture of *La. lactis* and is an effective antimicrobial against many Gram-positive bacteria (Branby-Smith, 1992). It also has some effectiveness against certain Gram-negative spoilage organisms and pathogens such as *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium (Martin-Visscher, Yoganathan, Sit, Lohans, & Vederas, 2011). Novel uses of nisin have been investigated; for example, nisin incorporated into biodegradable polylactic acid polymer film effectively controlled foodborne pathogens, including *Li. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Enteritidis, in culture media and in liquid foods, such as orange juice and liquid egg white (Jin & Zhang, 2008). In another study, the effect of nisin, added to zein film coatings on ready-to-eat chicken, on *Li. monocytogenes* was tested (Janes, Kooshesh, & Johnson, 2002). Chicken samples inoculated with *Li. monocytogenes* were dipped into zein with and without added nisin and stored at 4 °C or 8 °C for 24 day. At 4 °C, the growth of *Li. monocytogenes* was suppressed by 4.5−5.0 $\log_{10}$ CFU/g.

A commercial product named Alta 2341® has also come on the market. It is described by the manufacturer (Quest International) as a crude fermentation product of LAB, and the antimicrobial component was not specified by Quest. Schlyter, Degnan, Loeffelholz, Glass, and Luchansky (1993) postulated that Alta 2341 is pediocin AcH, a fermentation by-product of *P. acidilactici*. Rozum and Maurer (1997) determined that Alta 2341 extended cooked breast meat refrigerated shelf life up to 5 weeks. Szabo and Cahill (1999) determined that treatment of vacuum-packaged smoked fish with 1.0% Alta 2341 inhibited *Li. monocytogenes* growth for 21 day at 4 °C. Treatment of frankfurters stored at 4 °C with Alta 2341 effectively prolonged the time prior to growth of *Li. monocytogenes* for 7 weeks and reduced the growth of *Li. monocytogenes* for up to 12 weeks (Chen, Sebranek, Dickson, & Mendonca, 2004).

Although nisin, pediocin, and many other bacteriocins exhibit significant antimicrobial qualities, their use alone in a food may not ensure sufficient safety. Gram-negative bacteria do not represent target cells for bacteriocins, as they are protected by an outer membrane; therefore, it may be necessary to use bacteriocins in combination with other preservation methods. For example, nisin in combination with high-pressure homogenization was effective in obtaining a 5.0 $\log_{10}$ reduction of generic *E. coli* in apple and carrot juices (Pathanibul, Taylor, Davidson, & Harte, 2009). Combination treatments may also be appropriate for Gram-positive pathogens. Encapsulated nisin combined with low temperatures was applied to effectively control *Li. monocytogenes* in milk (da Silva Malheiros, Daroit, da Silveira, & Brandelli, 2010).

7.5 Use of LAB in the biopreservation of particular food products and as a biosanitizer

Consumer perceptions of food safety affect their food choices in unique ways. When a food or an ingredient is unfamiliar, consumers tend to amplify the risk; conversely, their perception of risk declines with familiar foods or ingredients (Grunert, 2005). Consumers are increasingly conscious of food contents and are more frequently reading labels; when they encounter names of additives that are difficult to pronounce and not familiar to them, they perceive these substances to present a higher risk (Song & Schwarz, 2009). LAB has the potential to provide a "clean label" desired by many consumers, and may represent a useful and effective strategy to prevent or reduce the incidence of pathogens, thus improving food safety and consumer health (Callaway et al., 2008).

The use of LAB as protective cultures may possess several advantages over the use of purified bacteriocins; the cultures may serve as the source of bacteriocins as well as a broad range of other antimicrobials including organic acids, carbon dioxide, ethanol, hydrogen peroxide, and diacetyl, as detailed in Chapter 6. These microorganisms may also exhibit antimicrobial activity against spoilage microorganisms, thus increasing shelf life. Therefore, LAB can be used as protective cultures to inhibit pathogens and/or prolong the shelf life of foods, or they may be useful as biosanitizers to reduce

or eliminate colonization of pathogens in an environment. The proper choice of LAB (either alone or in combination with other hurdles), as well as the selection and development of strains with best performance for each particular food, may greatly reinforce the competitiveness of biopreservation methods in the food industry. Recent application of LAB in nonfermented foods to increase safety or maintain quality is detailed in Table 7.1.

7.5.1 Poultry and meat

Maragkoudakis et al. (2009) screened 635 LAB of food origin for their potential application as protective cultures on chicken meat. The selected cultures exhibited antimicrobial activity against various pathogenic and spoilage bacteria, survived simulated food processing and gastrointestinal tract conditions, and were not antibiotic resistant or capable of hemolysis. Two chosen strains (*Enterococcus faecium* and *Lactobacillus fermentum*) were then applied on raw chicken meat to evaluate their protective ability against two common food pathogens, *Li. monocytogenes* and *Sa.* Enteritidis, and to identify potential contributions to spoilage. The protective cultures significantly reduced growth of the pathogens, and did not appear to have any detrimental effect on biochemical parameters related to spoilage of the chicken meat.

Lactobacillus sakei CECT 4808 and *Lactobacillus curvatus* CECT 904 T, known producers of bacteriocin-like substances, were inoculated individually or in combination on slices of beef. The samples were vacuum packaged and stored at refrigeration temperatures, and were assessed during a 28-day storage period for microbiological and chemical parameters, odor, and instrumental color. Samples inoculated with the *La. sakei* strain or the combination of the two strains yielded significantly lower spoilage microorganisms than those inoculated with the *La. curvatus* strain alone or the controls; in addition, *La. sakei* significantly improved chemical parameters (including lipid oxidation) and abnormal odor scores (Katikou, Ambrosiadis, Georgantelis, Koidis, & Georgakis, 2005).

7.5.2 Fruit and vegetables

In addition to the research on meat products, biopreservatives research has been conducted on minimally processed vegetables and fruits, such as prewashed and precut salads, and prepared fruit salads. Many of these ready-to-eat or ready-to-use salads do not contain preservatives, but the high water activity as well as the high number of cut surfaces with a resultant release of nutrients can provide ideal conditions for microbial growth, including pathogens (Hanning, Johnson, & Ricke, 2008; Hanning, Nutt, & Ricke, 2009).

Classical treatments, employing chlorine or ozone, very often fail to remove pathogens (Trias, Badosa, Montesinos, & Bañeras, 2008). Most of the strains applied as protective cultures in vegetables and fruits are LAB. Trias et al. (2008) isolated three strains of *Leuconostoc mesenteroides* from fresh fruits and vegetables and applied them as bioprotective cultures on intentionally wounded golden delicious apples and iceberg lettuce leaf cuts. They found that these strains were able to reduce the

Table 7.1 Use of lactic acid bacteria as biopreservatives

Food product	Culture employed	Target organism	References
Beef	*Lactobacillus sakei*	Spoilage	Katikou et al. (2005)
	Lactobacillus curvatus	Spoilage	Castellano, González, Carduza, and Vignolo (2010)
	La. curvatus	*Escherichia coli* O157:H7	Castellano, Belfiore, and Vignolo (2011)
Pork	*La. curvatus*	*Listeria monocytogenes*, *Staphylococcus aureus*	Castellano, Aristoy, Sentandreu, Vignolo, and Toldrá (2012)
Chicken	*Enterococcus faecium*, *Lactobacillus fermentum*	*Salmonella*, *Li. monocytogenes*	Maragkoudakis et al. (2009)
	Leuconostoc pseudomesenteroides	*Campylobacter jejuni, Li. monocytogenes*	Melero, Diez, Rajkovic, Jaime, and Rovira (2012)
Fish	*La. sakei*	Spoilage	Katikou, Ambrosiadis, Georgantelis, Koidis, and Georgakis (2007)
	Carnobacterium divergens	*Li. monocytogenes*	Brillet et al. (2005)
Onions	*En. faecium*, *Lactobacillus lactis*	*Listeria innocua*, Spoilage	Yang, Fan, Jiang, Doucette, and Fillmore (2012)
Lettuce	*L. lactis, L. plantarum, Pediococcus acidilactici*	*Li. monocytogenes*	Allende et al. (2007)
Apples	*Leuconostoc mesenteroides*	*Salmonella* Typhimurium, *E. coli*, *Li. monocytogenes*	Trias et al. (2008)

amount of *Sa.* Typhimurium and *E. coli* and to completely inhibit the growth of *Li. monocytogenes* without sensory or visual modifications of the product.

7.5.3 Seafood

The demand for minimally processed seafood has increased, thus raising considerable interest in biopreservation for ensuring safety and quality (Calo-Mata et al., 2008). In spite of several *in vitro* studies, very few commercial applications using LAB have appeared in seafood products, partially due to the organoleptic and nutritional quality of the food often being compromised and partially because bacteria that gave promising results *in vitro* were later shown to be ineffective when added to actual food products (Leroi, 2010). Brillet, Pilet, Prevost, Cardinal, and Leroi (2005) applied a culture of *Carnobacterium divergens* V41 to sterile cold-smoked salmon coinoculated with a mixture of *Li. monocytogenes* strains; the *C. divergens* was able to maintain the level of *Li. monocytogenes* at less than 50 CFU/g during 4 weeks of vacuum storage at 4 °C and 8 °C.

7.5.4 Biosanitation

The attachment of *Li. monocytogenes* to stainless steel, a material widely used in the food processing environment, increases the risk of cross contamination of *Li. monocytogenes* into food products (Wilks, Michels, & Keevil, 2006). Ndahetuye, Koo, O'Bryan, Ricke, and Crandall (2012) evaluated the ability of a LAB cocktail to inhibit the attachment of *Li. monocytogenes* to stainless steel. The LAB cocktail significantly reduced *Li. monocytogenes* population levels on stainless steel pretreated with LAB as compared with controls. The LAB cocktails also had a significant inhibitory effect on the attachment of *Li. monocytogenes* when both organisms were added to stainless steel at the same time. When stainless steel surfaces were contaminated first with *Li. monocytogenes*, then treated with the LAB cocktail, *Li. monocytogenes* was significantly displaced after a 24-h contact time. Thus, LAB cocktails such as this may play a role as a biosanitizer in the food industry.

7.6 Conclusions

Research on LAB has shown that a number of natural antimicrobials produced by these microorganisms are capable of playing an expanding role in meeting the increasing consumer demand for "clean labels" on their food products. The time seems opportune for increased commercial evaluation of LAB in potential novel roles as biopreservatives and biosanitizers.

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References

Allende, A., Martínez, B., Selma, V., Gila, M. I., Suárez, J. E., & Rodríguez, A. (2007). Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. *Food Microbiology*, *24*, 759−766.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*, 493−496.

Branby-Smith, F. M. (1992). Bacteriocins: applications in food preservatives. *Trends in Food Science and Technology*, *3*, 133−137.

Brashears, M. M., & Durre, W. A. (1999). Antagonistic action of *Lactobacillus lactis* toward *Salmonella* spp. and *Escherichia coli* O157:H7 during growth and refrigerated storage. *Journal of Food Protection*, *62*, 1336−1340.

Brashears, M. M., Reilly, S. S., & Gilliland, S. E. (1998). Antagonistic action of cells of *Lactobacillus lactis* toward *Escherichia coli* O157:H7 on refrigerated raw chicken meat. *Journal of Food Protection*, *61*, 166−170.

Braun, V., Schaller, K., & Wabl, M. R. (1974). Isolation, characterization, and action of colicin M. *Antimicrobial Agents and Chemotherapy*, *5*, 520−533.

Bredholt, S., Nesbakken, T., & Holck, A. (1999). Protective cultures inhibit growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in cooked, sliced, vacuum- and gas-packaged meat. *International Journal of Food Microbiology*, *53*, 43−52.

Bredholt, S., Nesbakken, T., & Holck, A. (2001). Industrial application of an antilisterial strain of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum-packaged meats. *International Journal of Food Microbiology*, *66*, 191−196.

Brillet, A., Pilet, M. F., Prevost, H., Cardinal, M., & Leroi, F. (2005). Effect of inoculation of *Carnobacterium divergens* V41, a bio-preservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. *International Journal of Food Microbiology*, *104*, 309−324.

Callaway, T. R., Edrington, T. S., Anderson, R. C., Harvey, R. B., Genovese, K. J., Kennedy, C. N., et al. (2008). Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Animal Health Research Reviews*, *9*, 217−225.

Calo-Mata, P., Arlindo, S., Boehme, K., de Miguel, T., Pascoal, A., & Barros-Velazquez, J. (2008). Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservation of aquatic food products. *Food and Bioprocess Technology*, *1*, 43−63.

Castellano, P., Aristoy, M. C., Sentandreu, M. A., Vignolo, G., & Toldrá, F. (2012). *Lactobacillus sakei* CRL1862 improves safety and protein hydrolysis in meat systems. *Journal of Applied Microbiology*, *113*, 1407−1416.

Castellano, P., Belfiore, C., & Vignolo, G. (2011). Combination of bioprotective cultures with EDTA to reduce *Escherichia coli* O157:H7 in frozen ground-beef patties. *Food Control*, *22*, 1461−1465.

Castellano, P., González, C., Carduza, F., & Vignolo, G. (2010). Protective action of *Lactobacillus curvatus* CRL705 on vacuum-packaged raw beef: effect on sensory and structural characteristics. *Meat Science*, *85*, 394−401.

Chen, C.-M., Sebranek, J. G., Dickson, J. S., & Mendonca, A. F. (2004). Use of pediocin (ALTA-2341) for control of *Listeria monocytogenes* on frankfurters. *Journal of Muscle Foods*, *15*, 35−56.

European Food Safety Authority, EFSA. (2013). *Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update)*. Available at http://www.efsa.europa.eu/en/efsajournal/doc/3449.pdf.

Gratia, A. (1946). Techniques sélectives pour la recherche systématique des germes antibiotiques. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales*, *140*, 1053−1055.

Grunert, K. G. (2005). Food quality and safety: consumer perception and demand. *European Review of Agricultural Economics*, *32*, 369−391.

Ham, J. S., Kim, H. S., Hong, K. H., Kim, J. G., Jeong, S. G., Chae, H. S., et al. (2003). Inhibitory activity of lactic acid bacteria against hazardous microbes. *Asian − Australasian Journal of Animal Sciences*, *16*, 1550−1554.

Hanning, I. B., Johnson, M. G., & Ricke, S. C. (2008). Precut prepackaged lettuce: a risk for listeriosis? *Foodborne Pathogens and Disease*, *5*, 731−746.

Hanning, I. B., Nutt, J. D., & Ricke, S. C. (2009). Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathogens and Disease*, *6*, 635−648.

Herody, C., Soyeux, Y., Hansen, E. B., & Gillies, K. (2010). The legal status of microbial food cultures in the European Union: an overview. *The European Food and Feed Law Review*, *5*, 258−269.

Hugas, M., Pagés, F., Garriga, M., & Monfort, J. M. (1998). Application of the bacteriocinogenic *Lactobacillus sakei* CTC494 to prevent growth of *Listeria* in fresh and cooked meat products packaged with different atmospheres. *Food Microbiology*, *15*, 639−650.

Immonen, N., & Karp, M. (2007). Bioluminescence-based bioassays for rapid detection of nisin in food. *Biosensors & Bioelectronics*, *22*, 1982−1987.

Janes, M. E., Kooshesh, S., & Johnson, M. G. (2002). Control of *Listeria monocytogenes* on the surface of refrigerated, ready-to-eat chicken coated with edible zein film coatings containing nisin and/or calcium propionate. *Journal of Food Science*, *67*, 2754−2757.

Jin, T., & Zhang, H. (2008). Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. *Journal of Food Science*, *73*, M127−M134.

de Jong, A., van Heel, A. J., Kok, J., & Kuipers, O. P. (2010). BAGEL2: mining for bacteriocins in genomic data. *Nucleic Acids Research*, *38*, W647−W651.

Kang, D., & Fung, D. Y. C. (1998). New methods for screening lactic acid bacteria that produce bacteriocin active against *Listeria monocytogenes* and for determining bacteriocin activity. *Journal of Rapid Methods and Automation in Microbiology*, *6*, 59−66.

Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., & Georgakis, S. A. (2005). Effect of *Lactobacillus*-protective cultures with bacteriocin-like inhibitory substances' producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. *Journal of Applied Microbiology*, *99*, 1303−1313.

Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., & Georgakis, S. A. (2007). Effect of *Lactobacillus* cultures on microbiological, chemical and odour changes during storage of rainbow trout fillets. *Journal of the Science of Food and Agriculture*, *87*, 477−484.

Koo, O. K., Eggleton, M., O'Bryan, C. A., Crandall, P. G., & Ricke, S. C. (2012). Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes* on frankfurters formulated with and without lactate/diacetate. *Meat Science*, *92*, 533−537.

Kuttner, A. G. (1966). Production of bacteriocines by group A streptococci with special reference to the nephritogenic types. *Journal of Experimental Medicine*, *124*, 279−291.

Leroi, F. (2010). Occurrence and role of lactic acid bacteria in seafood products. *Food Microbiology*, *27*, 698−709.

Leuschner, R. G. K., Robinson, T. P., Hugas, M., Cocconcelli, P. S., Richard-Forget, F., Klein, G., et al. (2010). Qualified presumption of safety (QPS): a generic risk assessment approach for biological agents notified to the European Food Safety Authority (EFSA). *Trends in Food Science and Technology*, *21*, 425−435.

Lindgren, S. E., & Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews*, *7*, 149−163.

Mallesha, R. S., Selvakumar, D., & Jagannath, J. H. (2010). Isolation and identification of lactic acid bacteria from raw and fermented products and their antibacterial activity. *Recent Research in Science and Technology*, *2*, 42−46.

Maragkoudakis, P. A., Mountzouris, K. C., Psyrras, D., Cremonese, S., Fischer, J., Cantor, M. D., et al. (2009). Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Salmonella enteritidis*. *International Journal of Food Microbiology*, *130*, 219−226.

Martin-Visscher, L. A., Yoganathan, S., Sit, C. S., Lohans, C. T., & Vederas, J. C. (2011). The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against Gram-negative bacteria in combination with EDTA treatment. *FEMS Microbiology Letters*, *317*, 152−159.

Meena, M. R., & Sethi, V. (1994). Antimicrobial activity of essential oils from spices. *Journal of Food Science Technology*, *31*, 68−70.

Melero, B., Diez, A. M., Rajkovic, A., Jaime, I., & Rovira, J. (2012). Behaviour of non-stressed and stressed *Listeria monocytogenes* and *Campylobacter jejuni* cells on fresh chicken burger meat packaged under modified atmosphere and inoculated with protective culture. *International Journal of Food Microbiology*, *158*, 107−112.

Milillo, S. R., Story, R. S., Pak, D., O'Bryan, C. A., Crandall, P. G., & Ricke, S. C. (2013). Antimicrobial properties of three lactic acid bacterial cultures and their cell free supernatants against *Listeria monocytogenes*. *Journal of Environmental Science and Health, Part B*, *48*, 63−68.

Moraes, P. M., Perin, L. M., Tassinari Ortolani, M. B., Yamazi, A. K., Viçosa, G. N., & Nero, L. A. (2010). Protocols for the isolation and detection of lactic acid bacteria with bacteriocinogenic potential. *LWT − Food Science Technology*, *43*, 1320−1324.

Moschetti, G., Villani, F., Blaiotta, G., Baldinelli, A., & Coppola, S. (1996). Presence of non-functional nisin genes in *Lactococcus lactis* subsp. *lactis* isolated from natural starters. *FEMS Microbiology Letters*, *145*, 27−32.

Ndahetuye, J. B., Koo, O. K., O'Bryan, C. A., Ricke, S. C., & Crandall, P. G. (2012). Role of lactic acid bacteria as a biosanitizer to prevent attachment of *Listeria monocytogenes* F6900 on deli slicer contact surfaces. *Journal of Food Protection*, *75*, 1429−1436.

Pathanibul, P., Taylor, T. M., Davidson, P. M., & Harte, F. (2009). Inactivation of *Escherichia coli* and *Listeria innocua* in apple and carrot juices using high pressure homogenization and nisin. *International Journal of Food Microbiology*, *129*, 316−320.

Rogers, A. H. (1974). Bacteriocin production and susceptibility among strains of *Streptococcus mutans* grown in the presence of sucrose. *Antimicrobial Agents and Chemotherapy*, *6*, 547−550.

Rogers, A., & Montville, T. (1991). Improved agar diffusion assay for nisin quantification. *Food Biotechnology*, *5*, 161−168.

Rozum, J. J., & Maurer, A. J. (1997). Microbiological quality of cooked chicken breasts containing commercially available shelf-life extenders. *Poultry Science*, *76*, 908−913.

Salvucci, E., Saavedra, L., & Sesma, F. (2007). Short peptides derived from the NH_2-terminus of subclass IIa bacteriocin enterocin CRL35 show antimicrobial activity. *Journal of Antimicrobial Chemotherapy*, *59*, 1102−1108.

Schlyter, J. H., Degnan, A. J., Loeffelholz, J., Glass, K. A., & Luchansky, J. B. (1993). Evaluation of sodium diacetate and ALTA™ 2341 on viability of *Listeria monocytogenes* in turkey slurries. *Journal of Food Protection*, *56*, 808–810.

da Silva Malheiros, P., Daroit, D. J., da Silveira, N. P., & Brandelli, A. (2010). Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk. *Food Microbiology*, *27*, 175–178.

Song, H., & Schwarz, N. (2009). If it's difficult to pronounce, it must be risky: fluency, familiarity, and risk perception. *Psychological Science*, *20*, 135–138.

Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*, *70*, 331–345.

Szabo, E. A., & Cahill, M. E. (1999). Nisin and ALTA 2341 inhibit the growth of *Listeria monocytogenes* on smoked salmon packaged under vacuum or 100% CO_2. *Letters in Applied Microbiology*, *28*, 373–377.

Tadesse, G., Ephraim, E., & Ashenafi, M. (2005). Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shameta, traditional Ethiopian fermented beverages, on some food-borne pathogens and effect of growth medium on the inhibitory activity. *Internet Journal of Food Safety*, *5*, 13–20.

Tagg, J. R., & McGiven, A. R. (1971). Assay system for bacteriocins. *Applied Microbiology*, *21*, 943.

Tramer, J., & Fowler, G. G. (1964). Estimation of nisin in foods. *Journal of the Science of Food and Agriculture*, *15*, 522–528.

Trias, R., Badosa, E., Montesinos, E., & Bañeras, L. (2008). Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. *International Journal of Food Microbiology*, *127*, 91–98.

U.S. Food and Drug Administration. (2011). *Federal food, drug and cosmetic act*. Available at http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/.

U.S. Food and Drug Administration. (2013). *Generally recognized as safe (GRAS)*. Available at http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/ucm2006850.htm.

Vera Pingitore, E., Hebert, E. M., Sesma, F., & Nader-Macías, M. E. (2009). Influence of vitamins and osmolites on growth and bacteriocin production by *Lactobacillus salivarius* CRL 1328 in a chemically defined medium. *Canadian Journal of Microbiology*, *55*, 304–310.

Virolainen, N., Guglielmetti, S., Arioli, S., & Karp, M. (2012). Bioluminescence-based identification of nisin producers – a rapid and simple screening method for nisinogenic bacteria in food samples. *International Journal of Food Microbiology*, *158*, 126–132.

de Vuyst, L. (1994). Nisin production variability between natural *Lactococcus lactis* subsp. *lactis* strains. *Biotechnology Letters*, *16*, 287–292.

Wilks, S. A., Michels, H. T., & Keevil, C. W. (2006). Survival of *Listeria monocytogenes* Scott A on metal surfaces: Implications for cross-contamination. *International Journal of Food Microbiology*, *111*, 93–98.

Yaakoubi, K., Benkerroum, N., Wiorowski, F., Sanson, F., Haydersah, J., & Chevallier, I. (2009). Development of a multiwell antagonistic activity assay for the detection of bacteriocin production by lactic acid bacteria. *Journal of Rapid Methods and Automation in Microbiology*, *17*, 32–45.

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*, 48. Available at http://dx.doi.org/10.1186/2191-0855-2-48.

Chitosan as an antimicrobial in food products

8

S. Zivanovic, R.H. Davis, D.A. Golden
University of Tennessee, Knoxville, TN, USA

8.1 Introduction

Chitin, (1→4)-2-acetamido-2-deoxy-β-D-glucan, is the second most abundant polysaccharide in nature, with estimated annual biosynthesis at a level of 10^9–10^{10} tons (Peter, 1995), and is a by-product of shellfish processing (Goosen, 1997; Skjak-Braek, Anthonsen, & Sandford, 1989). Commercially, chitosan is produced almost exclusively from chitin by alkali deacetylation. Theoretically, chitin is an acetylglucosamine polymer with all monomer units acetylated (100% degree of acetylation (DA)) and chitosan is a completely deacetylated product. However, chitosan is the designated name for the series of polymers with different ratios of glucosamine and *N*-acetyl glucosamine monomer units. Most commercial chitosans have less than 30% acetylated units (referred to as DA <30%) and a molecular weight (MW) between 100 and 1500 kDa. The presence of amino groups on glucosamine units results in its polycationic nature in acidified environment, which gives chitosan its unique characteristics.

Being a biodegradable, edible, and biocompatible natural polymer, chitosan has received a lot of attention for numerous applications in agriculture, the food industry, pharmacy, and biotechnology processes (Hirano, 1997; Knorr, 1984; Shahidi, Arachchi, & Jeon, 1999). Applications include wastewater purification (Knorr, 1991), chelation of metals (Muzzarelli, 1977), coating of seeds for improved yield and protection from fungal diseases (Hadwiger, Fristensky, & Riggleman, 1984), and drug delivery systems (Gupta & Ravi Kumar, 2000; Uhrich, Cannizzaro, Langer, & Shakesheff, 1999). Chitosan has been applied in production of biodegradable films and fibers, cell and enzyme immobilization matrices, healing-accelerating sutures and coverings, contact lenses, and artificial skin (Allan et al., 1984; Begin & Calsteren, 1999). It also has been used as a blood anticoagulant and feed additive and has been shown to possess antimicrobial properties. Chitosan is used to form gels, films, beads, and fibers (Guibal, 2004; Sankararamakrishnan, Dixit, Iyengar, & Sanghi, 2006; Zivanovic, Li, Davidson, & Kit, 2007), in waste water management as a chelator (Shahidi et al., 1999), and in medicine as wound dressings (Koide, 1998). Chitosan has been researched for use in the food industry in various fields, including as an antimicrobial, antioxidant, a thickening agent in beverages, a clarifying agent in juices, and as a packaging material (Devlieghere, Vermeulen, & Debevere, 2004; Shahidi et al., 1999; Xie, Xu, & Liu, 2001). The health care industry uses about 65% of total chitosan, agriculture 12%, waste and water treatments 7%, the food and beverage industry

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6%, and immobilization and biotechnology 5% (Knorr, 1991; Li, Dunn, Grandmaison, & Goosen, 1997).

Chitosan is approved as a food additive in Korea and Japan (Hirano, 1997; Japan Food Chemical Research Foundation, 2011; KFDA, 2011; No, Meyers, Prinyawiwatkul, & Xu, 2007; No, Park, Lee, & Meyers, 2002). The European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies has permitted the use of health claims such as "can help in the reduction of body weight" and "stimulates regulation of cholesterol" for chitosan as food constituent (EFSA, 2011). In the United States, chitosan has been sold for years as a dietary supplement, was approved by the Environmental Protection Agency (EPA) to be used as a biopesticide (Docket No. EPA-HQ-OPP-2007-0566-0019), and the Food and Drug Administration (FDA) recognized *Aspergillus niger*—derived chitosan as safe for use as a processing aid in alcoholic beverages (FDA, 2011).

8.2 Overview of antimicrobial activity of chitosan

Chitosan possesses antibacterial and antifungal properties and has been extensively studied as a potential natural antimicrobial agent in the pharmaceutical, cosmetic, and food industries (Goosen, 1997; Ravi Kumar, 2000; Shahidi et al., 1999). Chitosan exhibits activity against some strains of fungi (Allan & Hadwiger, 1979; Hirano & Nagao, 1989; Kenedra & Hadwiger, 1984; Roller & Covill, 1999; Stossel & Leuba, 1984), yeasts (Roller & Covill, 1999; Sudarshan, Hoover, & Knorr, 1992), and bacteria (Chen, Liau, & Tsai, 1998; Roller & Covill, 2000; Wang, 1992). Chitosan inhibits the growth of several important foodborne bacteria including *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, and *Lactobacillus fructivorans* (Papineau, Hoover, Knorr, & Farkas, 1991; Roller & Covill, 2000; Sudarshan et al., 1992; Wang, 1992). However, reported minimum inhibitory concentrations (MICs) for both bacteria and yeasts vary widely, from 0.01% to 5.0%, depending on polymer characteristics, pH, temperature, and the presence of interfering substances such as proteins and fats (Benhabiles et al., 2012; Chen et al., 1998; Rhoades & Roller, 2000; Roller & Covill, 1999; Sudarshan et al., 1992; Tao, Qian, & Xie, 2011; Tsai & Su, 1999; Tsai, Wu, & Su, 2000). Considerable variations between genera, species, and strains, and between the same strains under different environmental conditions, have been documented (Table 8.1).

8.3 Mechanism of action

8.3.1 In vitro

When applied *in vitro*, in systems such as processed food or microbiological media, the antimicrobial activity of chitosan could be the result of one or a combination of several proposed mechanisms, depending on the type of microorganism and the characteristics of the chitosan.

Table 8.1 Antimicrobial effect of chitosan against foodborne microorganisms

Microorganism	Chitosan concentration (mg/l)	Effect	Reported specifics	References
Aeromonas hydrophila	1000	MLC	DP 24	Tsai et al. (2000)
Escherichia coli	100		DP 16	
Listeria monocytogenes	150		DP 16	
Pseudomonas aeruginosa	200		DP 5	
Salmonella Typhimurium	1500		DP 5	
Shigella dysenteriae	200		DP 5	
Staphylococcus aureus	50		DP 29	
A. hydrophila	500	MIC		Chen et al. (1998)
E. coli	100		DDA 69%	
P. aeruginosa	200			
S. Typhimurium	>2000			
S. dysenteriae	200			
L. monocytogenes	100			
S. aureus	100			
Bacillus cereus	1000			

Continued

Table 8.1 Continued

Microorganism	Chitosan concentration (mg/l)	Effect	Reported specifics	References
E. coli	600	MIC	MW 685 kDa	Jeon et al. (2001)
S. typhi	600			
P. aeruginosa	2500			
E. coli	800	MIC	MW 470 kDa	No, Park, Lee, and Meyers (2002)
P. fluorescens	800			
S. Typhimurium	800			
S. aureus	800			
L. monocytogenes	800			
B. cereus	500			
B. megaterium	500			
Lactobacillus plantarum	1000			
L. brevis	1000			
L. bulgaricus	>1000			
Bacillus sp.	50	MIC	59−1671 kDa	No, Park, Lee, Hwang, et al. (2002b)
Enterobacter (Cronobacter) sakazakii	50			
S. epidermidis	1000−10,000	Inhibition		Allen et al. (1984)
S. aureus	10,000	Inhibition		
P. aeruginosa	10,000	Inhibition		
Candida tropicalis	10,000	Inhibition		
S. pyogenes	10,000	Slight effect		
E. coli	10,000	Slight effect		

Botrytis cinerea	3000	Inhibition		Fang, Li, and Shin (1994)
Rhizopus stolonifer	5000	76% Inhibition		
Aspergillus niger	5000	41% Inhibition		
A. parasiticus	5000	63% Inhibition		
Saccharomyces cerevisiae	200	Inactivation		Papineau et al. (1991)
E. coli				
A. flavus	10,000	Not affected	Chitosan glutamate	Roller and Covill (1999)
Cladosporium	10,000	Not affected	25 °C	
cladosporioides	10,000	Not affected		
Penicillium	1000−5000	MIC		
aurantiogriseum	1000−5000	MIC		
Byssochlamys spp.	100	Inhibited		
Mucor racaemosus	5000	Inhibited		
Zygosaccharomyces bailii				
Saccharomycodes ludwigii				
Salmonella Enteritidis	5000	Not affected	pH 7	Roller and Covill (2000)
Lactobacillus fructivorans	100	Inhibited	pH 6 and 3.4	
Z. bailii	500	Not affected	pH 5.5/3.4	
S. Typhimurium	2000	Inactivation (1−5 logs)	pH 5.8	Sudarshan et al. (1992)
Z. bailii	100		Apple juice	
Clostridium perfringens	250	MLC	DDA 95−70%	Tsai and Hwang (2004)
Lactobacillus acidophilus	500	MLC	DDA 95%	

Continued

Table 8.1 Continued

Microorganism	Chitosan concentration (mg/l)	Effect	Reported specifics	References
Candida lambica *Cryptococcus lumiculus* *Photobacterium phosphoreum* *Pseudomonas fluorescens* *Enterobacter aeromonas* *B. cereus* *Brochothrix thermosphacta* *L. monocytogenes* *Lactobacillus plantarum* *Lactobacillus curvatus* *Pediococcus acidilactici*	400 80 80 80 60 60 80 200 500−750 750 500	MIC	43 kDa; DDA 94%	Devlieghere et al. (2004)
E. coli *S. aureus*	50−180 400−>1600	MIC	6.9−22.2 kDa	Chen, Kung, Chen, and Lin (2010)
E. coli *S. aureus*	40−120 480−>1600	MIC	6.9−24.8 kDa DDA 80% and 92%	Lin, Lin, and Chen (2009)
S. aureus (3 strains)	440−1600	MIC	LMW; DDA 92%	Chen et al. (2012)
E. coli *Pseudomonas aeruginosa*	100 500	MIC	12 kDa; DDA 80%	Benhabiles et al. (2012)

S. aureus	300			
S. Typhimurium	>1000			
B. subtilis	100			
B. cereus	100			
Vibrio cholerae	100			
Shigella dysenteriae	100			
Enterobacter agglomerans	100			
Prevotella melaninogenica	100			
Bacteroides frangilis	60			
B. cereus	63 100	MIC MBC	56.8 kDa; DDA 84%	Mellegard, From, Christensen, and Granum (2011)
S. aureus	380 (750)	MIC (MBC)	HMW; DDA >85%	Tao et al. (2011)
E. coli	750 (750)			
L. monocytogenes	3000 (>3000)			
S. Enteritidis	3000 (300,000)			
B. cereus	3000 (>3000)			
P. aeruginosa	3000 (>3000)			

DDA, degree of deacetylation; DP, degree of polymerization; HMW, high molecular weight; LMW, low molecular weight; MBC, minimum bactericidal concentration; MIC, minimal inhibitory concentration; MLC, minimal lethal concentration; MW, molecular weight.

Antibacterial properties of chitosan have been widely investigated and confirmed (Coma, Deschamps, & Martial-Gros, 2003; Dutta, Tripathi, Mehrotra, & Dutta, 2009; Kong et al., 2008; Liu, Du, Wang, & Sun, 2004; Zivanovic, Basurto, Chi, Davidson, & Weiss, 2004).

Several mechanisms have been proposed to explain the effect of chitosan on bacteria. The most commonly accepted antibacterial mechanism is based on the electrostatic interactions between the polycationic charged chitosan and the negatively charged components at the cell surface, for example, the anionic cell wall glycans and/or proteins or phospholipids in the cytoplasmic membrane (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Young & Kauss, 1983). Depending on the type and location of the interaction, this may have various effects. Binding of chitosan with the cell wall anionic macromolecules apparently forms an impermeable layer around the cell, which can prevent the transport of nutrients to and from the cell (Choi et al., 2001; Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008). Interaction with cell membrane constituents may alter permeability, resulting in leakage of intracellular electrolytes, glucose, enzymes, and other proteinaceous cytoplasmic material (Coma et al., 2003; Dutta et al., 2009; Fang et al., 1994; Helander et al., 2001; Kong et al., 2008; Leuba & Stossel, 1986; Liu et al., 2004; Tao et al., 2011; Tsai & Su, 1999; Young & Kauss, 1983).

In a study by Liu et al. (2004), *S. aureus* and *E. coli* were treated with 0.5% and 0.25% 78-kDa chitosan, which resulted in a 1.0 $\log_{10}$ reduction after 5 min, complete inactivation of *E. coli* after 120 min, and no change in *S. aureus* after 120 min. The cells were examined by transmission electron microscopy, and *E. coli* had an altered and chitosan-covered outer membrane but no damage to the inner membrane. *S. aureus*, however, showed leakage of intracellular material, and new cells without membranes or cell walls on the outside had formed. Another study by Helander et al. (2001) treated *E. coli* and *Salmonella* Typhimurium with 0.025% of 85% DDA chitosan, which altered the outer membrane of the cell and formed a layer around *E. coli.* Chitosan seems to have stronger bactericidal effects against Gram-positive than against Gram-negative bacteria (Dagry, Reddy, Benhamou, & Arul, 2000; Helander et al., 2001; No, Park, Lee, & Meyers, 2002). This is probably because of the difference in the net electronegativity of the cell wall between Gram-positive and Gram-negative bacteria. Gram-positive bacteria have an electronegative surface of the cell wall originating from phosphoryl groups of teichoic and teichuronic acids. In Gram-negative species, however, the outer lipopolysaccharide membrane suppresses the net charges by providing partial hydrophobicity and reducing the exposure of unsubstituted acidic groups. Capsules and other forms of extracellular films of some bacteria are composed of oligosaccharides, polysaccharides, proteoglycans, and/or proteins, which can additionally affect surface charge.

In addition to environmental factors and species of bacteria, MW and the degree of deacetylation of chitosan affect the binding intensity of polycationic chitosan to bacterial surfaces. Zheng and Zhu (2003) suggested that chitosan interrupts the physiological activities of the cell but affects Gram-positive and Gram-negative cells differently. They proposed that while chitosan with a higher MW forms a polymer "shield" to prevent nutrients from entering and leaving the cell on Gram-positive

organisms, lower-molecular-weight chitosan enters the Gram-negative cell, binding to compounds in the cytoplasm, which causes flocculation and disrupts physiological processes.

Another possible antibacterial mechanism of chitosan is through chelation of essential nutrients needed for growth (Dutta et al., 2009). Kong et al. (2008) found that chitosan microspheres (1456 kDa) were chelating Mg^{2+} of the *E. coli* outer membrane, causing destabilization of the cell. Further, chelating ions from the cell wall may disrupt the organization of wall polymers and interfere with the activity of wall-bound enzymes (Kong, Chen, Xing, & Park, 2010). The fourth proposed mechanism of action occurs by inhibiting messenger RNA and protein synthesis by penetrating the cell of the microorganism and binding with DNA (Hadwiger, Kendra, Fristensky, & Wagoner, 1986; Sudarshan et al., 1992). To penetrate the nucleus, chitosan molecules have to have a low MW. Studies showed that a heptamer exhibited maximum antifungal activity, which decreased with the chain length, although saccharides with three or fewer units were also inactive (Hirano & Nagao, 1989; Kenedra & Hadwiger, 1984).

Similar mechanisms have been proposed for the *antifungal activity* of chitosan. As for antibacterial effect, the most commonly accepted mechanism involves the interaction of chitosan with the cell wall or with the cell plasma membrane, causing leakage of intracellular material (El Ghaouth, Arul, Asselin, & Benhamou, 1992; Liu, Tian, Meng, & Xu, 2007). Thus, El Ghaouth et al. (1992) found that chitosan caused morphological changes and leakage of amino acids due to the accumulation of chitosan in the cell wall of *Rhizopus stolinfer* and *Botritis cinerea*. Guerrero, Tognon, and Alzamora (2005) found 0.1% chitosan inhibited the growth and caused about a 1-log reduction in the first few minutes of treatment by causing structural defects in the cell wall of *Saccharomyces cerevisiae*. Seyfarth, Schliemann, Elsner, and Hipler (2008) noticed that 120-kDa chitosan hydrochloride disrupted the plasma membrane in *Candida albicans*, *Candida krusei*, and *Candida glabrata*, leading to permeable cells. Another mechanism involves the chelation of Ca^{2+}, Zn^{2+}, and other essential minerals needed for growth (Cuero, Duffus, Osuji, & Pettit, 1991; Roller & Covill, 1999). A study by Roller and Covill (1999) found that chitosan reduced the growth rates of *Mucor racemosus* by making Ca^{2+} and other essential minerals unavailable. When cultures of wood-degrading fungi (*Sphaeropsis sapinea* and *Trichoderma harzianum*) were treated with chitosan, increased chitosan concentration (from 0.01% to 0.15%) had a direct effect on the increase in K^{+} leakage; alterations in the plasma membrane; severity of fungal morphology changes including excessive branching, vacuolation, and reduced hyphal diameter; increase in hydrogen peroxide accumulation; and decrease of superoxide formation (Singh, Vesentini, Singh, & Daniel, 2008). However, the authors noted that the primary target of chitosan treatment on these fungi seemed to be the plasma membrane. Hadwiger and Loschke (1981), however, applied 0.0002% of chemically cleaved chitosan to pea plants to protect them against *Fusarium solani* and found that chitosan reduced *Fusarium* growth by interfering with its DNA.

Most of the *antiviral activity* of chitosan has been studied on plants before harvest, although there is recent interest in applying chitosan to ensure the safety

of production after harvest (Bautista-Banos et al., 2006; Cota-Arriola, Cortez-Rocha, Burgos-Hernandez, Ezquerra-Brauer, & Plascencia-Jatomea, 2013; Davis, Zivanovic, Davidson, & D'Souza, 2012; Friedman & Juneja, 2010; Su, Zivanovic, & D'Souza, 2009). One proposed postharvest mechanism is that chitosan causes structural damage to the virus (Kochkina & Chirkov, 2000a; Kochkina, Surgucheva, & Chirkov, 2000). Using electron microscopy, Kochkina et al. (2000) saw chitosan causes a loss in viral tail fibers with receptors and viral sheath contraction, exposing the DNA of phage T2 and 1−97A. Another mechanism proposes that chitosan interacts with the negative charge of the viral capsids (Kochkina & Chirkov, 2000b; Su et al., 2009). Su et al. (2009) found 0.7% 53-kDa chitosan caused a 1.7-log reduction of bacteriophage MS2, which could be due to the negatively charged bacteriophage MS2, with an isoelectric point of 3.9 (Langlet, Gaboriaud, & Gantzer, 2007), binding to the positively charged chitosan. The third proposed mechanism involves inhibiting phage infection (Kochkina, Pospieszny, & Chirkov, 1995). A study found that chitosan inhibits the infection of *E. coli* by bacteriophages T2 and T7, possibly by attaching to the viral particles (Kochkina et al., 1995). Still another mechanism proposed chitosan interferes with a step in the replication process (Chirkov, 2002; Kochkina & Chirkov, 2000a). Kochkina and Chirkov (2000a) found chitosan to be efficient at inhibiting phage replication in *E. coli*, which the authors thought to be due to the interruption of the intracellular reproduction of the phages.

8.3.2 In vivo

While antimicrobial application after harvest is important to ensure safety and reduce spoilage, treatments with chitosan before harvest are important for improving crop yield. Because most plant-pathogenic fungi contain chitin or chitosan in their cell walls, plants react to chitosan application similar to a fungal attack: by activating defensive responses. This includes the synthesis of various phytoalexins, enzymes, hydrogen peroxide, and phenolics and the accumulation of lignin in cell walls (Fajardo, McCollum, McDonald, & Mayer, 1998; Iriti et al., 2010; Li, Chen, & Yanbin, 2009; Vander, Varum, Domard, El Gueddari, & Moerschbacher, 1998). Enhancing its defense mechanism, a plant becomes less susceptible to infection by fungi, bacteria, or viruses. Concentrations of chitosan as low as 18 mg/l have been shown to elicit synthesis of phytoalexins and stimulate accumulation of phenolics and lignin by host plants (Allan & Hadwiger, 1979; Reddy, Arul, Angers, & Couture, 1999). When harvested fruits were wounded and inoculated with pathogen fungi, those treated with chitosan had less fungal growth than fruits not treated with chitosan. Wu, Zivanovic, Draughon, Conway, and Sams (2005) noticed significantly smaller lesions on harvested apples caused by *Botrytis cinerea* and *Penicillium expansum* if the apples were pretreated with chitosan. When pear fruits were inoculated with *Alternatia kikuchiana* Tanaka or *Physalospora piricola* Nose, application of 350-kDa chitosan in the wound before inoculation reduced mycelial growth and spore germination and caused a significant increase in peroxidase activity in fruits (Meng, Yang, Kennedy, & Tian, 2010). Similarly, chitosan-treated wheat seeds have improved seed germination and increased resistance against plant-pathogenic fungi (Kulikov,

Chirkov, Il'ina, Lopatin, & Varlamov, 2006; Reddy et al., 1999; Reddy, Belkacemi, Corcuff, Castaigne, & Arul, 2000). On tobacco necrosis virus, 0.15% 76-kDa chitosan induced abscisic acid production, resulting in plant resistance to the virus (Iriti & Faoro, 2008).

Chitosan has been widely investigated in *animal and human studies* because of its ability to bind lipids and thus possibly assist in weight loss (Hernandez-Gonzales, González-Ortiz, Martínez-Abundis, & Robles-Cervantes, 2010; Ikeda et al., 1993; Karadeniz, Karagozlu, Pyun, & Kim, 2011; Maezaki et al., 1993; Neyrinck et al., 2009). It also has been evaluated and commercialized for its hemostatic and wound-healing properties (Archana, Dutta, & Dutta, 2013; Busilacchi, Gigante, Mattioli-Belamonte, Manzotti, & Muzzarelli, 2013; Croisier & Jerome, 2013; Gerentes, Vachoud, Doury, & Dornad, 2002; Howling et al., 2001; HemCon Medical Technologies, Inc., Portland, OR). Although wound-healing efficiency is enhanced by chitosan's antimicrobial activity (Burkatovskaya et al., 2006; Koide, 1998; Okamoto et al., 1995), this topic exceeds the focus of this chapter. From the standpoint of animal health and food safety, chitosan has been investigated as a feed additive and immunity enhancer. In one of the first studies evaluating the effect of dietary chitosan on intestinal microflora, Tanaka et al. (1997) found that mice fed with a casein diet containing 5% chitosan gradually lost appetite over 4 weeks and consumed less food compared with the control group, resulting in reduced weight, whereas 0.5% chitosan in the diet did not have an effect on appetite and weight. Similarly, 0.5% dietary chitosan did not affect fecal microflora. However, 5% dietary chitosan caused a higher level of anaerobes (0.5 $\log_{10}$) and lower counts of facultative anaerobes (1.0 $\log_{10}$), *Bifidobacterium* (0.5 $\log_{10}$), and *Lactobacillus* (2.0 $\log_{10}$) and did not significantly alter the number of *Enterobacteriaceae*. Comparing chitosan's activity against *Clostridium perfringens*, *Bifidobacterium*, and *Lactobacillus*, Tsai and Hwang (2004) reported much stronger *in vitro* efficiency against *C. perfringens* than against the probiotics, whereas overall antimicrobial activity after chitosan ingestion *in vivo* was insignificant. When 6-day-old broiler chickens were fed a diet containing 3% chitosan adipate and infected with *Salmonella* Gallinarum, clinical symptoms and anatomicopathological changes were weak compared with infected chickens not fed chitosan (Balicka-Ramisz, Wojtasz-Pajak, Pilarczyk, & Ramisz, 2007). However, compared with the uninfected group (the control group not fed chitosan and that gained 12.5% weight during the experiment), chitosan-treated chicks gained only 10−10.5% weight. To enhance the immune system and resistance of koi fish (*Cyprinus carpio koi*) to *Aeromonas veronii*, fish were fed diets containing probiotics (*Bacillus coagulans*), prebiotics (0.2% 5-kDa chitosan), or both (Lin et al., 2012). A combination diet containing *B. coagulans* and chitosan resulted in the highest final weight, specific growth rate, total leucocyte count, respiration burst activity, phagocytic activity, lysozyme activity, and superoxide dismutase activity and the highest disease resistance to *A. veronii* (36.9% average mortality compared with 67.5% for the controls). Similarly, 80% mortality rate of kelp grouper (*Epinephelus bruneus*) infected with *Vibrio alginolyticus* decreased to 30% and 25% when 1% and 2% chitosan was added to the fish diet (Harikrishnan, Kim, Balasundaram, & Heo, 2012).

Immunostimulating adjuvant effects of chitosan have been explored in human and veterinary medicine (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Koide, 1998; Van der Lubben, Verhoef, Borchard, & Junginger, 2001). For example, administration of live Newcastle disease vaccine by the oculonasal route to 1-day-old chickens had significantly better results when the vaccine was given with 0.5% chitosan (Rauw et al., 2010). Chitosan enhanced the antigen-specific cell-mediated immune response in the spleen and shortened the local cellular immune response in the digestive tract.

8.4 Effects of molecular structure

Chitosan is polymolecular and polydisperse, as are many other polysaccharides. This means that molecules could vary in structure and MW. Ideally, chitosan is a linear β-1,4-homopolymer consisting of glucosamine units. Chitin, the parent molecule, is theoretically a homopolymer consisting of only β-1,4-acetylglucosamine units, in which acetyl groups are attached to amino group at the C_2 atom of glucosamine. However, polysaccharides with 70% or more 2-acetamido-2-deoxy-β-D-glucose units (acetylglucosamine), and, consequently, with less than 30% 2-amino-2-deoxy-β-D-glucose (glucosamine), are also considered to be chitins. Polysaccharides with a minimum of 70% glucosamine (and therefore a maximum of 30% acetylglucosamine) units are called chitosan. Chitosan is commercially produced by deacetylating chitin. Concentrated alkali solutions randomly cleave the bond between amide and the acetyl group on C_2 of the acetylglucosamine unit [$-NH-(CO-CH_3)$] leaving the distribution of amino and acetyl groups in the chitosan molecule with no particular order. Chitosan has both reactive amino and hydroxyl groups, and its properties change with environmental conditions. It has a pK_a of approximately 6.3, and it is soluble in aqueous acidic solutions at pH 6 or less, at which it behaves as a polycationic polymer. The physical and chemical characteristics of chitosan solutions are affected by the MW and degree of acetylation of the molecule, pH, ionic strength, and temperature of the solution (Pa & Yu, 2001; Rinaudo, Milas, & Dung, 1993).

Considering the available literature, it may be speculated that the larger and more deacetylated the chitosan molecule (larger MW and higher DDA as compared to DA), the greater the possibility that it interacts with the cell wall of Gram-positive bacteria, causing a "shielding effect." Less deacetylated chitosan, however, may interact better with lipopolysaccharides in the outer membrane of Gram-negative bacteria. Further, if the MW of such chitosan is sufficiently low, the molecule may be able to pass through the cell wall and interact with the cell membrane, causing leakage of cytoplasmic material.

8.4.1 Molecular weight

It has been generally accepted that chitosan is a more effective as an antimicrobial against Gram-positive (MIC 100−1250 mg/l) than Gram-negative bacteria (MIC 100−10,000 mg/l). However, conflicting results regarding the correlation of the MW and antimicrobial activity of chitosan have been reported. Comparing the

efficiency of chitosans of various MWs and DDA on the growth and germination of *Bacillus cereus*, Mellegard et al. (2011) declared that 56.8- and 98.3-kDa chitosans with 84% DDA were significantly more efficient than one with a 10.6-kDa MW, whereas there was no difference in activity against *B. cereus* between chitosans with a 19.6- and 163-kDa MW when DDA was 52%. However, Zivanovic et al. (2004) found that 150-kDa chitosan (75−85% DDA) inhibited the Gram-positive *Listeria monocytogenes* and Gram-negative *S.* Typhimurium better than 5-kDa chitosan lactate. Joen, Park, and Kim (2001) compared chitosan with an average MW of 685 kDa with three levels of oligomers: high, with an MW distribution between 7 and 24 kDa; medium, between 1.5 and 6 kDa; and low, consisting of oligosaccharides with a degree of polymerization in the range of pentamer to heptamer. The authors found that growth inhibition of all tested microorganisms (*Bacillus subtilis*, *E. coli*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Streptococcus faecalis*, and *Salmonella typhi*) increased with increasing MW and that an MW of at least 10 kDa is required for effective inhibition of Gram-positive and Gram-negative bacteria. Uchida, Izume, and Ohtakara (1989) enzymatically hydrolyzed chitosan and produced oligomers with total reducing sugar (TRS) content from 50 to 590 mg/g. They found that native, nonhydrolyzed chitosan was twice as effective against *Fusarium* spp. than oligomers. The native chitosan and the mildly hydrolyzed chitosan (TRS content 50 mg/g) had similar effects on *E. coli*, but the hydrolysate was more active against *P. aeruginosa*, *B. subtilis*, and *S. aureus.* However, the most highly degraded hydrolysate (TRS content 590 mg/g) did not have any antimicrobial effect, even at a concentration of 10,000 mg/l. Chitosans with a 28−1671-kDa MW were more efficient than chitosan oligomers with a 1- to 22-kDa MW and had an overall stronger bactericidal effect toward Gram-positive (*L. monocytogenes*, *S. aureus*, *B. cereus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *L. bulgaricus*) than toward Gram-negative bacteria (*E. coli*, *Pseudomonas fluorescens*, *S. typhimurium*, and *Vibrio parahaemolyticus*) (No, Park, Lee, Hwang, & Meyers, 2002; No, Park, Lee, & Meyers, 2002). No, Kim, Lee, Park, and Prinyawiwatkul (2006) also noticed that 0.05% of 1110- and 2025-kDa chitosans were sufficient to completely suppress the growth of *L. monocytogenes* and were generally slightly more effective against Gram-positive (*L. monocytogenes* and *S. aureus*) than against Gram-negative bacteria (*E. coli* and *S.* Enteritidis). Further, chitosan with an MW of 400 kDa displayed a weaker antibacterial effect compared with larger molecules of chitosan glutamate and chitosan lactate, both with an MW in the range of 1000−1500 kDa (Sudarshan et al., 1992). Tao et al. (2011) explained that higher efficiency of high-molecular-weight chitosan toward Gram-positive *S. aureus* (MIC 380 mg/l) compared with Gram-negative *P. aeruginosa* (MIC 3000 mg/l) was due to the increased permeability of cell membranes.

Lin et al. (2009), however, found that chitosans with a low MW (6−24.8 kDa) and 80% and 92% DDA had a stronger antibacterial effect toward *E. coli* than toward *S. aureus.* Also, results of work by Chen et al. (2010) indicate that chitosan hydrolysates with an MW of 6.9−22.2 kDa were more efficient against *E. coli* than against *S. aureus* and that antibacterial function was exerted by both surface and permeation

effects. Devlieghere et al. (2004) determined that Gram-negative bacteria were very susceptible to the effects of 43-kDa, 94% DDA chitosan, whereas Gram-positive bacteria varied greatly in response to the chitosan; some were inhibited and others were less affected. Furthermore, Gerasimenko, Avdienko, Bannikova, Zueva, and Varlamov (2004) reported that chitosans with a MW between 5 and 27 kDa effectively inhibited both Gram-positive and Gram-negative bacteria. Earlier, Tsai et al. (2000) found that, when tested against *Aeromonas hydrophila*, *E. coli*, *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium*, *Shigella dysenteriae*, *S. aureus*, *Vibrio cholerae*, and *Vibrio parahemolyticus*, the maximum lethal concentration of native chitosan was in the 50−1000 mg/l range, whereas for a chitosan oligosaccharides mixture (degree of polymerization (DP) $\leq$8) the maximum lethal concentration was 5−29 mg/l. Examining the effect of chitosan with an MW of 3, 50, and 1000 kDa, Li et al. (2009) found that all three chitosans caused cellular membrane damage in *E. coli*, but 50-kDa chitosan was the most efficient. Similarly, Liu, Guan, Yang, Li, and Yao (2001) found that antimicrobial activity increased when the MW of chitosan increased from 5 to 91.6 kDa and decreased when the MW further increased to 1080 kDa. Evaluating the larger number of chitosans with an MW in the range of 55−500 kDa (DDA 80%), Liu et al. (2006) reported that lower-MW chitosans (<155 kDa) were more efficient against *E. coli* than those with a higher MW. Furthermore, they noticed that, regardless of MW, chitosan in concentrations less than 20 ppm (<0.002%) could promote the growth of *E. coli*.

It seems that chitosan with a high MW forms an impermeable layer (film) around Gram-positive bacteria through interactions with cell wall compounds (teichoic and lipoteichoic acid), whereas low-MW chitosans are more efficient toward Gram-negative bacteria by passing through the cell wall (lacking the teichoic acids) and interacting with the cell membrane, disrupting the cell metabolism. As an example, Zheng and Zhu (2003) found that as MW increased from less than 5−305 kDa, the antibacterial activity against Gram-positive bacteria (*S. aureus*) increased and decreased against Gram-negative bacteria (*E. coli*). The authors found the lower MW (<5 kDa) inhibited *E. coli* by entering the cell and interrupting physiological activities of the cell, whereas the higher MW (166 and 305 kDa) inhibited *S. aureus* by forming a film around the cell that suppressed nutrient transport.

The effect of the MW of chitosan against viruses has mostly been studied indirectly *in vivo*, as chitosan increases plants' resistance to viral attack. Chirkov, Surgucheva, Gamzazade, Abdulabekov, and Pospeshny (1998) found that as the MW of chitosan increased from 3 to 50 kDa, the resistance of bean plants to bean mild mosaic virus increased. However, another study found the opposite trend for the same virus. This study used chitosan with MWs of 1.2, 2.2, 10.1, 30.3, and 40.4 kDa to find that bean plants' resistance to bean mild mosaic virus increased as MW decreased (Kulikov et al., 2006). A study by Su et al. (2009) evaluated the direct effects of chitosan of different MWs on human norovirus surrogates, which showed a trend of increasing antiviral activity. The authors found that 53-kDa chitosan reduced viral titers of bacteriophage MS2 and FCV-F9 by 1.70 and 4.21 $\log_{10}$ plaque-forming units/ml, respectively, whereas 5-kDa chitosan reduced titers by only 0.98 and 1.41 $\log_{10}$ plaque-forming units/ml, respectively.

It should be noted that MWs reported in most publications were those declared by manufacturers and were not analyzed before antimicrobial experiments. In addition, the MW of chitosan is usually determined by measuring intrinsic viscosity, assuming uniform MW among all molecules. Although significant polydispersity of chitosan molecules is well known, it was rarely taken into account or reported. A vague definition of "low," "medium," and "high" MW further complicates the issue. For example, authors who evaluate the effect of chitosans with an MW in the range of, for example, 5−55 kDa, declare chitosan with a 55-kDa MW as "high MW," whereas authors who work with chitosans in the range of, for example, 150−650 kDa, label 150-kDa chitosan as "low MW." Thus, to help understand chitosan efficiency, it is reasonable to encourage the publication of manuscripts only if all details of the chitosan used are provided.

8.4.2 Degree of deacetylation

The antimicrobial activity of chitosan apparently increases with degree of deacetylation (DDA) (Kong et al., 2010; Roberts, 1992). Molecules with a DDA $\geq$ 70% are referred to as "chitosan," and molecules with a DDA of approximately 50% are commonly labeled as "water-soluble chitosan" (solubility in water can be achieved by various modifications of native chitosan, although the simplest one is by decreasing the MW and adjusting the DDA to 50%). The higher the DDA, the more monomer units in the polymer carry an available, easily protonated amino group at C_2 ($R-NH_2$) instead of an acetamido group ($R-NH-C(O)-CH_3$). The pK_a of chitosan is 6.3, at which half of the amino groups are protonated, providing a positive charge. With each unit of pH decrease, there is increase of 1 $\log_{10}$ in protonated groups on chitosan. One mechanism of antimicrobial activity—maybe the most accepted—is based on electrostatic interaction between the positive charge of chitosan and the negative charge of microbial cell, and an increase in the charge density of chitosan should result in more efficient activity. This was experimentally confirmed. Thus, chitosan with a DDA of 75% reduced the optical density by 0.02 in *E. coli* suspensions within 2 h of application, whereas more deacetylated chitosan exhibited prolonged activity over 16 h, resulting in decreased optical density for 0.04, 0.05, and 0.07 at DDAs of 83%, 90%, and 96%, respectively (Liu et al., 2001). Similarly, growth and germination of *B. cereus* was suppressed more with chitosans with a DDA $\geq$84% compared with a DDA 52% (Mellegard et al., 2011). Chitosan with a 95% DDA caused more intensive leakage of alkaline phosphatase, glucose-6-phosphate dehydrogenase, and nucleotides from *S. aureus* and *E. coli* compared with chitosan with a 75% DDA (Chung & Chen, 2008). Using highly deacetylated chitosan with 98% DDA, Tsai and Su (1999) reported that *E. coli* cells in the late exponential phase were most sensitive, followed by stationary cells and cells in the mid-exponential phase. Gilbert, Evans, Evans, Duguid, and Brown (1991) reported that the negative charge of *E. coli* cells progressively increased during the exponential phase but then decreased. This suggests that the susceptibility of *E. coli* cells to chitosan depends on the electronegativity of the cell surface in addition to the charge of the chitosan molecule.

In general, bacterial surfaces are negatively charged because of the presence of charged wall polysaccharides and membrane macromolecules, for example, peptidoglycans and phospholipids. The degree of this charge can be determined based on the electrostatic mobility of cells in an electric field, and is defined as the zeta potential. Although the zeta potential varies at the strain and species levels, it also changes with the age of the culture. Thus, zeta potential cannot be assigned to a microorganism species as an absolute value. However, measurements at strictly defined conditions offer useful quantifications for comparison (Table 8.2). The zeta potential of a single chitosan molecule in a solution has not yet been measured because of the small size of the molecule. However, chitosan clusters in acidic solutions have been evaluated, and a surface charge of +43 mV has been recorded (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997).

Chen et al. (2010) reported that two strains of *E. coli* were more susceptible to chitosan (MW 6.9−22.2 kDa) than were three strains of *S. aureus* because of the

Table 8.2 Surface charge of several bacteria and yeast strains

Strain	Zeta potential (mV)
Listeria monocytogenes	~−55
Staphylococcus aureus BCRC 10451	−5.99
S. aureus BCRC 10780	−4.26
S. aureus BCRC 10781	−5.47
Streptococcus salivarius GB 16/2	−19.6
S. salivarius GB 24/9	−11.7
Staphylococcus epidermidis GB 9/6	−28.3
Staphylococcus hominis GB S1	−15.8
Escherichia coli wild-type	~−15
E. coli BCRC 10304	−13.7
E. coli BCRC 10675	−7.84
E. coli O157:H7	~−3
Pseudomonas aeruginosa IFO3455	−21.4
Salmonella Minnesota	−1.3
Candida albicans GB 1/2	−15.4
C. ablicans GB 8/1	−17.3
Candida tropicalis GB 9/9	−8.3
C. tropicalis GB 19/4	−4.9

Sources: Asai, Iwamoto, and Watanabe (1998); Briandet, Meylheuc, Maher, and Bellon-Fontaine (1999); Busscher, Geertsema-Doornbusch, and van der Mei (1997); Chen et al. (2010); Lytle, Rice, Johnson, and Fox (1999); Poelstra et al. (2000).

Table 8.3 Yeast growth in apple juice prevented by chitosan

Yeasts	Hours with no visible growth of yeast in apple juice as affected by chitosan concentration			
	0%	0.005%	0.01%	0.05%
Candida krusei	12	24	48	96
Saccharomyces cerevisiae	12	48	96	96
Zygosaccharomyces baillii	24	48	72	144

difference in zeta potential of the bacterial surface (−7.84 and −13.7 mV for *E. coli* and −4.26 to −5.99 mV for *S. aureus*). Furthermore, the difference in zeta potential between the two *E. coli* strains were sufficient for a corresponding susceptibility to chitosan. In our work, we determined the zeta potential of three yeast species to be −12.07, −20.52, and −25.82 for *C. krusei* NRRL 7179, *Saccharomyces cerevisiae* KE 162, and *Zygosaccharomyces bailii* NRRL 7256, respectively (Michael, 2005; Michael, Zivanovic, & Golden, 2004). The yeast cell surface charge was not affected by culture age up to the late stationary phase but had less negative charge once the cells entered the early decline phase. Medium-MW chitosan exhibited the strongest antimicrobial activity toward *Z. bailii* and the least activity against *C. krusei*. Even the lowest tested concentration of chitosan (0.005%) suppressed the growth of *Z. bailii* and *S. cerevisiae* for 2 days in test media. The highest tested concentration (0.05%) inhibited the growth of *Z. bailii* for 6 days, whereas the growth of *S. cerevisiae* and *C. krusei* was suppressed for 4 days at 25 °C. When tested in apple juice at 22 °C, chitosan suppressed the growth of yeast and significantly prolonged shelf life (Michael, 2005; Michael, Rajpal, Golden, & Zivanovic, 2005) (Table 8.3).

8.5 Effects of environmental conditions

Application in real food systems usually requires higher concentrations of chitosan than in nutrient media or buffers because of interactions with proteins, lipids, salts, and other constituents that can alter chitosan activity. Thus, although the MIC of a chitosan oligomers mixture for *E. coli*, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus* in nutrient broth at 37 °C was between 5 and 29 mg/l, chitosan oligomers did not affect milk microflora under the same conditions. However, higher concentrations (0.24% and 0.48%) of chitosan oligomers at a lower temperature (4 °C) did reduce the cell count and extended the shelf life of raw milk by at least 4 days (Tsai et al., 2000). Darmadji and Izumimoto (1994) showed that 1% chitosan was necessary for just a 1.0- to 2.0-$\log_{10}$ reduction of pseudomonads, staphylococci, and total bacteria count in minced beef patties, whereas lower concentrations (0.2% and 0.5%) had no effect on the spoilage flora. On the other hand, fresh strawberries and bell peppers dipped

in acidic chitosan solutions and inoculated with *B. cinerea* or *Rhizopus stolonifer* were equally resistant to spoilage as fruits treated with a conventional fungicide (El Ghaouth, Arul, Ponnampalam, & Boulet, 1991; El Ghaouth, Arul, Wilson, & Benhamou, 1997). Growth of eight yeast species in apple juice at 25 °C depended on chitosan concentration, temperature, and pH, but 0.1−5 g/l chitosan glutamate was sufficient for growth inhibition of all species, with Z. *bailii* being the most sensitive (Roller and Covill, 1999).

Most authors agree that the antimicrobial activity of chitosan increases with increased concentration (Liu et al., 2001; Wang, 1992). However, in some cases a dose−response relationship could not be demonstrated. For example, Knowles and Roller (2001) found that higher concentrations of chitosan glutamate (2.0%) were, in some cases, less biocidal than lower concentrations (0.5% and 1.0%), and Papineau et al. (1991) showed that 0.02% chitosan lactate was more effective against *E. coli* than 0.1% and 0.5%. Similarly, the antibacterial action of chitosan glutamate and chitosan lactate were very similar against *Moraxella* spp., *Brevibacterium linens*, *Enterobacter aerogenes*, *Pediococcus acidilactici*, *Proteus morganii*, *S.* Typhimurium, *E. coli*, and *S. aureus* and were more effective at 100 mg/l than 2000 or 5000 mg/l (Sudarshan et al., 1992).

Electrostatic interactions between chitosan molecules and bacterial walls are inhibited in an environment with high *ionic strength* containing 0.15 M sodium chloride (Briandet et al., 1999). Similarly, an increase in the ionic strength of sodium chloride caused a several-fold reduction in *E. coli* flocculation with chitosan. However, ionic strengths above 0.5 M weakened the repulsive forces in the bacterial wall and caused aggregation without any chitosan present (Strand, Varum, & Ostgaard, 2003). Tsai and Su (1999) hypothesized that Na^+ (0.1 M) might complex with chitosan and, accordingly, reduce chitosan activity against *E. coli*. Divalent cations (10 and 25 mM) had more pronounced efficacy and reduced antimicrobial activity of chitosan in the order $Ba^{2+} > Ca^{2+} > Mg^{2+}$. Apparently, metal ions bind with chitosan, forming complexes (ligands) through the involvement of OH and NH_3^+ groups. Although there is a possibility of the formation of a salt−chitosan complex, numerous data confirm the activity of chitosan in real food systems regardless of the presence of various salts.

Tsai and Su (1999) found that *temperature* directly effects the antibacterial activity of chitosan. When inoculated in phosphate buffer at pH 6.0, the bactericidal activity of 0.15 mg/l chitosan increased from 4 to 37 °C. At 25 and 37 °C, viable *E. coli* declined 6.0 $\log_{10}$, but only 4.0 and 3.0 $\log_{10}$ when incubated at 15 and 4 °C, respectively. The authors speculated that stress at low temperatures might alter cell surface characteristics and decrease the number of binding sites for chitosan. When chitosan was added to a 20% oil-in-water emulsion at pH 4.5, both *L. monocytogenes* and *S.* Typhimurium were more susceptible to chitosan at 10 °C than at 25 °C (Zivanovic et al., 2004). Similarly, Briandet et al. (1999) found that *L. monocytogenes* grown at 8 °C had significantly less surface electronegativity than when grown at 15, 20, and 37 °C. They speculated that the reduced charge at 8 °C was the result of synthesis of acclimation proteins and fewer carboxylic groups in the cell wall composition. In contrast, Tsai et al. (2000) found that 0.24% or 0.48%

oligosaccharides (DP $\leq$8) in raw milk reduced mesophilic and psychrotrophic bacteria by at least three logs at 4 °C but had no inhibitory effects if the milk was kept at 37 °C.

The antimicrobial activity of chitosan is also affected by pH. Chitosan's p*K*a is 6.3, and acidic conditions (pH $<$ pK_a) allow its solubility. In aqueous acetic acid solutions with a pH $>$3.5, chitosan molecules behave like flexible random coil polymers, whereas at a pH $<$2.0 they behave like rigid rods because of high intramolecular electrostatic repulsion. Because antimicrobial applications are generally evaluated at pH ranging between 3 and 6, it is assumed that chitosan molecules freely move through solution and bend around a microbial cell. As the pH of an environment decreases, the positive charge on a chitosan molecule increases, possibly providing more sites for electrostatic interaction with negatively charged microbial cells. However, the increased charge density on the molecule reduces its flexibility and potential to bend around the cell surface. Thus, it seems that the optimum pH for chitosan activity depends on the molecule itself (its DDA and MW) as well as of the surface charge of the microbial cell. Liu et al. (2001) reported that chitosan expressed a maximum inhibitory effect at pH 6.3. At neutral pH or higher (pH 7−8) chitosan was inactive, whereas in highly acid solution (pH $\sim$3) activity was moderate. Lower antimicrobial activity of chitosan at pH $<$3 was explained by the pK_a of wall peptidoglycans ($4 <$ p$K_a <$ 5) and the very low pK_a of the phosphate groups in the phosphodiester bridges of cell wall teichoic acids (p$K_a <$ 2.1) in Gram-positive species (Rijnaarts, Norbe, Lyklema, & Zehnder, 1995). Thus, the negative charge of a Gram-positive bacterial wall is reduced below pH 4 and diminished at pH $<$ 2, resulting in less antimicrobial efficiency of chitosan molecules. The outer membrane of Gram-negative bacteria, however, composed mainly of lipopolysaccharides (Weidenmaier & Peschel, 2008), carries less charge and thus may be more susceptible to chitosans with a lower DDA ($<$80−85%), especially at higher pH (pH $\sim$ pK_a). In addition, it may be speculated that chitosans with a higher MW would act more on the surface of bacteria, whereas oligomers would be able to penetrate into the cytoplasm. Helander et al. (2001) were the first to show that chitosan with a DDA of approximately 85% binds to the outer membrane of Gram-negative bacteria (at pH 5.3), speculating that the binding was primarily due to electrostatic interactions. However, insertion of chitosan into dipalmitoyl-phosphatidylglycerol monolayer films prepared to simulate negatively charged bacterial cell membranes was not only electrostatic based but also included nonelectrostatic interactions, including hydrophobic and hydrogen bonds (Krajewska, Wydro, & Kyzilol, 2013). However, when membranes were prepared with dipalmitoyl-phosphatidylcholine (such as zwitterion) and cholesterol (hydrophobic), the interaction was mainly hydrophobic and through hydrogen bonds. Although the dependence on temperature and pH was less pronounced than with dipalmitoyl-phosphatidylglycerol, in which the interaction was enhanced at a higher temperature (between 5 and 37 °C) and lower pH (between 3.5 and 6.0), the interaction with dipalmitoyl-phosphatidylcholine or cholesterol was promoted by higher temperature and higher pH (Krajewska, Kyzilol, & Wydro, 2013).

8.6 Current applications and future trends

Although chitosan has been used as a food additive in Korea and Japan (Japan Food Chemical Research Foundation, 2011; KFDA, 2011) and as a biopesticide (EPA, 2008), processing aid (FDA, 2011), dietary supplement, and in various forms of hemostatic wound dressings in the United States and around the world, it still does not have a true generally regarded as safe (GRAS) status. Interestingly, a leading chitosan producer has filed a GRAS request for chitosan three times but has always withdrawn the application (GRN 073 filed January 31, 2001, and withdrawn January 29, 2002; GRN 170 filed April 25, 2005, and withdrawn October 31, 2005; and GRN 443 filed August 21, 2012, and withdrawn February 11, 2013). The chitosan producer's requests were for shrimp-derived chitosan to be used as a direct additive either to food in general or to designated foods, including meat and poultry. The intended application was as an antimicrobial agent, emulsifier, nutrient supplement, processing aid, and/or stabilizer and thickener. However, another company's request for GRAS status (GRN 397) for their fungal-derived chitosan to be used as a processing aid in alcoholic beverages was approved in 2011 (FDA, 2011). The chitosan is allowed to be used at 10−500 g/100 L during the processing of wine, beer, cider, or spirits "for microbiological stabilization, removal of contaminants, and/or clarification of the alcoholic beverage" (FDA, 2011). Because a vast number of scientific publications claim benefits of potential use of chitosan as a food additive, it may be expected that chitosan will gain GRAS status in the future. One of the possible concerns over the use of shrimp-derived chitosan in food is potential allergy problems. Because a major source of chitosan is shellfish waste, and because shellfish is one of the most common allergens, there is a concern that chitosan may act as an allergen. However, Gray, Hutcheson, and Slavin (2004) determined that it was safe for people with shellfish allergies to use glucosamine supplements because it is the proteins of the flesh of the fish, and not the shell, that cause the reaction. Furthermore, the extraction and preparation of chitosan with hot concentrated sodium hydroxide should remove all proteins, including those that may be potential allergens, and the product should not be considered as part of the living organism that biosynthesized it (Muzzarelli, 2010).

In addition to being used as a processing aid and food additive, there is potential for chitosan to be used as a coating and packaging material. For this purposes, chitosan may be used in its native form, chemically modified, or added to synthetic polymers. Chemical modifications to increase its antimicrobial efficiency, improve stability, resistance to swelling, water vapor and gas permeability, as well as to introduce primary antioxidant properties, have been explored (Alves & Mano, 2008; Jayakumar, New, Tokura, & Tamura, 2007; Rivero, Garcia, & Pinotti, 2010; Sashiwa & Aiba, 2004; Schreiber, Zivanovic, Bozell, & Hayes, 2013; Vartiainen, Ratto, Lantto, Nattinen, & Hurme, 2008). Blending chitosan with synthetic polymers has been shown to improve the biodegradability and antimicrobial properties of "standard" packaging material without significantly affecting price (Li, Zivanovic, Davidson, & Kit, 2010, 2011; Massouda, Visioli, Green, & Joerger, 2012; Omura, Renbutsu, Morimoto,

Salmoto, & Shigemasa, 2003; Sunikumar, Francis, Thachil, & Sujith, 2012; Zhang, He, Liu, & Qia, 2009; Zivanovic et al., 2007).

While it can be expected that chitosan will be used more in commercial products, it is certain that extensive research efforts to define the exact mechanism of chitosan's biological activities and structure−function relationship will continue. For this purpose, encouraging authors to always provide detailed specification of chitosan used—at the minimum the MW and DDA—is highly desirable. Furthermore, a standardized method for determining the antimicrobial activity of chitosan films needs to be developed and accepted by the scientific community to meaningfully compare results.

References

Allan, C. R., Altman, L. C., Bensinger, R. E., Ghosh, D. K., Hirabayashi, Y., Neogi, A. N., et al. (1984). Biomedical application of chitin and chitosan. In J. P. Zikiakis (Ed.), *Chitin, chitosan, and related enzymes* (pp. 119−133). New York, NY: Academic Press.

Allan, C. R., & Hadwiger, L. A. (1979). The fungicidal effect of chitosan on fungi of varying cell wall composition. *Experimental Mycology*, *3*, 285−287.

Alves, N. M., & Mano, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. *International Journal of Biological Macromolecules*, *43*, 401−414.

Archana, D., Dutta, J., & Dutta, P. K. (2013). Evaluation of chitosan nano dressing for wound healing: characterization, *in vitro* and *in vivo* studies. *International Journal of Biological Macromolecules*, *57*, 193−203.

Asai, Y., Iwamoto, K., & Watanabe, S. (1998). The effect of the lipid A analog E5531 on phospholipid membrane properties. *FEBS Letters*, *438*, 15−20.

Balicka-Ramisz, A., Wojtasz-Pajak, A., Pilarczyk, B., & Ramisz, A. (2007). The effect of chitosan on body weight and protection against *Salmonella gallinarum* infection in broiler chickens. *Archiv Tierzucht Dummerstorf*, *50*, 288−293.

Bautista-Banos, S., Hernandez-Lauzardo, A. N., Velazquez-del Valle, M. G., Hernandez-Lopez, M., Barka, E. A., Bosquez-Molina, E., et al. (2006). Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop Protection*, *25*(2), 108−118.

Begin, A., & Calsteren, M. R. (1999). Antimicrobial films produced from chitosan. *International Journal of Biological Macromolecules*, *26*, 63−67.

Benhabiles, M. S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., & Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloids*, *29*, 48−56.

Briandet, R., Meylheuc, T., Maher, C., & Bellon-Fontaine, M. N. (1999). *Listeria monocytogenes* Scott A: cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. *Applied and Environmental Microbiology*, *65*(2), 5328−5333.

Burkatovskaya, M., Tegos, G. P., Swietlik, E., Demidova, T. N., Castano, A. P., & Hamblin, M. R. (2006). Use of chitosan bandage to prevent fatal infections developing from highly contaminated wounds in mice. *Biomaterials*, *27*(22), 4157−4164.

Busilacchi, A., Gigante, A., Mattioli-Belamonte, M., Manzotti, S., & Muzzarelli, R. A. A. (2013). Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydrate Polymers*, *98*, 665−676.

Busscher, H. J., Geertsema-Doornbusch, G. I., & van der Mei, H. C. (1997). Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *Journal of Biomedical Materials Research*, *34*, 201−209.

Calvo, P., Remunan-Lopez, C., Vila-Jato, J. L., & Alonso, M. J. (1997). Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *Journal of Applied Polymer Science*, *63*, 125−132.

Chen, C. S., Liau, W. Y., & Tsai, G. J. (1998). Antibacterial effects of *N*-sulfonated and *N*-sulfobenzoyl chitosan and applications to oyster preservation. *Journal of Food Protection*, *61*, 1124−1128.

Chen, L. C., Chiang, W. D., Chen, W. C., Chen, H. H., Huang, Y. W., Chen, W. J., et al. (2012). Influence of alanine uptake on *Staphylococcus aureus* surface charge and its susceptibility to two cationic antibacterial agents, nisin and low molecular weight chitosan. *Food Chemistry*, *135*, 2397−2403.

Chen, L. C., Kung, S. K., Chen, H. H., & Lin, S. B. (2010). Evaluation of zeta potential difference as an indicator for antibacterial strength of low molecular weight chitosan. *Carbohydrate Polymers*, *82*, 913−919.

Chirkov, S. N. (2002). The antiviral activity of chitosan (review). *Applied Biochemistry and Microbiology*, *38*, 1−8.

Chirkov, S. N., Surgucheva, N. A., Gamzazade, A. I., Abdulabekov, I. M., & Pospeshny, H. (1998). Comparative efficiency of chitosan derivatives at inhibition of plants virus infection. *Doklady Akademii Nauk*, *360*, 271−273.

Choi, B. K., Kim, K. Y., Yoo, Y. J., Oh, S. J., Choi, J. H., & Kim, C. Y. (2001). In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *International Journal of Antimicrobial Agents*, *18*(6), 553−557.

Chung, Y. C., & Chen, C. Y. (2008). Antibacterial characteristics and activity of acid-soluble chitosan. *Bioresourse Technology*, *99*, 2806−2814.

Coma, V., Deschamps, A., & Martial-Gros, A. (2003). Bioactive packaging materials from edible chitosan polymer − antimicrobial activity assessment on dairy-related contaminants. *Journal of Food Science*, *68*, 2788−2792.

Cota-Arriola, O., Cortez-Rocha, M. O., Burgos-Hernandez, A., Ezquerra-Brauer, J. M., & Plascencia-Jatomea, M. (2013). Controlled release matrices and micro/nanoparticles of chitosan with antimicrobial potential: development of new strategies for microbial control in agriculture. *Journal of the Science of Food and Agriculture*, *93*(7), 1525−1536.

Croisier, F., & Jerome, C. (2013). Chitosan-based biomaterials for tissue engineering. *European Polymer Journal*, *49*, 780−792.

Cuero, R. G., Duffus, E., Osuji, G., & Pettit, R. (1991). Aflatoxin control in preharvest maize − effects of chitosan and 2 microbial agents. *Journal of Agricultural Science*, *117*, 165−169.

Dagry, M. K., Reddy, B., Benhamou, N., & Arul, J. (2000). *Antimicrobial action of chitosan on post-harvest storage bacteria*. Dallas, TX: Institute of Food Technologists 59th Ann. Mtg. Abstract 65C-24.

Darmadji, P., & Izumimoto, M. (1994). Effect of chitosan in meat preservation. *Meat Science*, *38*, 243−254.

Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan − a versatile semi-synthetic polymer in biomedical applications. *Pogress in Polymer Science*, *36*, 981−1014.

Davis, R., Zivanovic, S., Davidson, P. M., & D'Souza, D. (2012). Effectiveness of chitosan on the reduction of enteric viral surrogates. *Food Microbiology*, *32*, 57–62.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antibacterial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703–714.

Dutta, P. K., Tripathi, S., Mehrotra, G. K., & Dutta, J. (2009). Perspectives for chitosan based antimicrobial films in food applications. *Food Chemistry*, *114*, 1173–1182.

Eaton, P., Fernandes, J. C., Pereira, E., Pintado, M. E., & Malcata, F. X. (2008). Atomic force microscopy study of the antibacterial effects of chitosans on *Escherichia coli* and *Staphylococcus aureus*. *Ultramicroscopy*, *108*(10), 1128–1134.

EFSA. (2011). Scientific opinion on the substantiation of health claims related to chitosan and reduction in body weight (ID 679, 1499), maintenance of normal blood LDL-cholesterol concentrations (ID 4663), reduction of intestinal transit time (ID 4664) and reduction of inflammation (ID 1985) pursuant to Article 13(1) of Regulation (EC0 No 1924/2006. *EFSA Journal*, *9*(6), 2214. European Food Safety Authority (EFSA), Parma, Italy.

El Ghaouth, A., Arul, J., Asselin, A., & Benhamou, N. (1992). Antifungal activity of chitosan on post-harvest pathogens: induction of morphological and cytological alterations in Rhizopus stolonifer. *Mycological Research*, *96*, 769–779.

El Ghaouth, A., Arul, J., Ponnampalam, R., & Boulet, M. (1991). Chitosan coating effect on storability and quality of fresh strawberries. *Journal of Food Science*, *56*, 1618–1620.

El Ghaouth, A., Arul, J., Wilson, C., & Benhamou, N. (1997). Biochemical and cytochemical aspects of the interactions of chitosan and *Botrytis cinerea* in bell pepper fruit. *Postharvest Biology and Technology*, *12*, 183–194.

EPA. (2008). *Chitin and chitosan. Final Registration Review Decision. Case 6063*. Docket Number EPA-HQ-2007-0566. Biopesticides and Pollution Prevention Division. The United States Environmental Protecting Agency (EPA). Available at http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2007-0566. Accessed on 08.01.13.

Fajardo, J. E., McCollum, T. G., McDonald, R. E., & Mayer, R. T. (1998). Differential induction of proteins in orange flavedo by biologically based elicitors and challenged by *Penicillium digitatum* Sacc. *Biological Control*, *13*(3), 143–151.

Fang, S. W., Li, C. J., & Shin, D. Y. C. (1994). Antifungal activity of chitosan and its preservative effect on low-sugar candied kumquat. *Journal of Food Protection*, *56*, 136–140.

FDA. (2011). *Agency response letter GRAS notice no. GRN 000397*. CFSAN/Office of Food Additive Safety.

Friedman, M., & Juneja, V. K. (2010). Review of antimicrobial and antioxidative activities of chitosans in food. *Journal of Food Protection*, *73*(9), 1737–1761.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. Y., & Varlamov, V. P. (2004). Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms. *Applied Biochemistry and Microbiology*, *40*, 253–257.

Gerentes, P., Vachoud, L., Doury, J., & Dornad, A. (2002). Study of chitosan-based gel as injectable material in periodontal surgery. *Biomaterials*, *23*, 1295–1302.

Gilbert, P., Evans, D. J., Evans, E., Duguid, I. G., & Brown, M. R. W. (1991). Surface characteristics and adhesion of *Escherichia coli* and *Staphylococcus epidermis*. *Journal of Applied Bacteriology*, *71*, 72–77.

Goosen, M. F. A. (1997). *Applications of chitin and chitosan*. Lancaster, PA: Technomic Publishing Co., Inc.

Gray, H. C., Hutcheson, P. S., & Slavin, R. G. (2004). Is glucosamine safe in patients with seafood allergy? *Journal of Allergy and Clinical Immunology*, *114*, 459–460.

Guerrero, S., Tognon, M., & Alzamora, S. M. (2005). Response of *Saccharomyces cerevisiae* to the combined action of ultrasound and low weight chitosan. *Food Control*, *16*, 131–139.

Guibal, E. (2004). Interactions of metal ions with chitosan-based sorbents: a review. *Separation and Purification Technology*, *38*, 43–74.

Gupta, K. C., & Ravi Kumar, M. N. V. (2000). Drug release behavior of beads and microgranules of chitosan. *Biomaterials*, *21*, 1115–1119.

Hadwiger, L. A., Fristensky, B., & Riggleman, R. C. (1984). Chitosan, a natural regulator in plant-fungal pathogen interaction, increases crop yields. In J. P. Zikakis (Ed.), *Chitin, chitosan, and related enzymes* (pp. 291–302). New York: Academic Press.

Hadwiger, L. A., Kendra, D. F., Fristensky, B. W., & Wagoner, W. (1986). Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. In R. A. A. Muzzarelli, C. Jeuniaux, & G. Gooday (Eds.), *Chitin in nature and technology* (pp. 209–214). New York: Plenum.

Hadwiger, L. A., & Loschke, D. C. (1981). Molecular communication in host-parasite interactions – hexosamine polymers (chitosan) as regulator compounds in race-specific and other interactions. *Phytopathology*, *71*, 756–762.

Harikrishnan, R., Kim, J. S., Balasundaram, C., & Heo, M. S. (2012). Immunomodulatory effects of chitin and chitosan enriched diets in *Epinephelus bruneus* against *Vibrio alginolyticus* infection. *Aquaculture*, *326–329*, 46–52.

Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology*, *71*, 235–244.

Hernández-González, S. O., González-Ortiz, M., Martínez-Abundis, E., & Robles-Cervantes, J. A. (2010). Chitosan improves insulin sensitivity as determined by the euglycemic-hyperinsulinemic clamp technique in obese subjects. *Nutrition Research*, *30*(6), 392–395.

Hirano, S. (1997). Application of chitin and chitosan in the ecological an environmental fields. In M. F. A. Goosen (Ed.), *Applications of chitin and chitosan* (pp. 31–54). Lancaster, PA: Technomic Publishing Co., Inc.

Hirano, S., & Nagao, N. (1989). Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agricultural and Biological Chemistry*, *53*, 3965–3066.

Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M., & Wood, E. J. (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. *Biomaterials*, *22*, 2959–2966. http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm287638.htm.

Ikeda, I., Sugano, M., Yoshida, K., Sasaki, E., Iwamoto, Y., & Hatano, K. (1993). Effects of chitosan hydrolysates on lipid absorption and on serum and liver lipid concentrations in rats. *Journal of Agricultural and Food Chemistry*, *41*, 431–435.

Iriti, M., & Faoro, F. (2008). Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV). *Plant Physiology and Biochemistry*, *46*, 1106–1111.

Iriti, M., Giulia, C., Sara, V., Ilaria, M., Soave, C., Fico, G., et al. (2010). Chitosan-induced ethylene-independent resistance does not reduce crop yield in bean. *Biological Control*, *54*, 241–247.

Japan Food Chemical Research Foundation. (2011). *List of existing food additives*. Complied and published by the Ministry of Health and Welfare on April 16, 1996. Available at http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add. Effective of May 6, 2011. Accessed on 08.01.13.

Jayakumar, R., New, N., Tokura, S., & Tamura, H. (2007). Sulfated chitin and chitosan as novel biomaterials. Review *International Journal of Biological Macromolecules*, *40*, 175–181.

Joen, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Karadeniz, F., Karagozlu, M. Z., Pyun, S. Y., & Kim, S. K. (2011). Sulfation of chitosan oligomers enhances their anti-adipogenic effect in 3T3-L1 adipocytes. *Carbohydrate Polymers*, *86*(2), 666–671.

Kenedra, D. F., & Hadwiger, L. A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal of *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. *Experimental Mycology*, *8*, 276–281.

KFDA. (2011). *Korea food additive code*. Korea Food and Drug Administration (KFDA). Available at http://www.mfds.go.kr/fa/ebook/egongjeon_intro.html; http://fa.kfda.go.kr/standard/egongjeon_standard_view.jsp?SerialNo=130&GoCa=2&currPage=1&stext=chitosan Accessed on 08.01.13.

Knorr, D. (1984). Use of chitinous polymers in food. *Food Tehnology*, *38*(1), 85–94, 97.

Knorr, D. (1991). Recovery and utilization of chitin and chitosan in food processing waste management. *Food Tehnology*, *45*(1), 114–122.

Knowles, J., & Roller, S. (2001). Efficacy of chitosan, carvacrol, and a hydrogen peroxide-based biocide against foodborne microorganisms in suspension and adhered to stainless steel. *Journal of Food Protection*, *64*(10), 1542–1548.

Kochkina, Z. M., & Chirkov, S. N. (2000a). Effect of chitosan derivatives on the reproduction of coliphages T2 and T7. *Microbiology*, *69*, 208–211.

Kochkina, Z. M., & Chirkov, S. N. (2000b). Influence of chitosan derivatives on the development of phage infection in the *Bacillus thuringiensis* culture. *Microbiology*, *69*, 217–219.

Kochkina, Z. M., Pospieszny, H., & Chirkov, S. N. (1995). Inhibition by chitosan of productive infection of the t-series bacteriophages in an *Escherichia coli* culture. *Mikrobiologia*, *64*, 211–215.

Kochkina, Z. M., Surgucheva, N. A., & Chirkov, S. N. (2000). Inactivation of coliphages by chitosan derivatives. *Microbiology*, *69*, 212–216.

Koide, S. S. (1998). Chitin-chitosan: properties, benefits and risks. *Nutrition Research*, *18*, 1091–1101.

Kong, M., Chen, X. G., Liu, C. S., Liu, C. G., Meng, X. H., & Yu, L. J. (2008). Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. *Colloids and Surfaces B: Biointerfaces*, *65*, 197–202.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. *International Journal of Food Microbiology*, *144*, 51–63.

Krajewska, B., Kyzilol, A., & Wydro, P. (2013). Chitosan as a subphase disturbant of membrane lipid monolayers. The effect of temperature at varying pH: II. DPPC and cholesterol. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *434*, 359–364.

Krajewska, B., Wydro, P., & Kyzilol, A. (2013). Chitosan as a subphase disturbant of membrane lipid monolayers. The effect of temperature at varying pH: I. DPPG. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *434*, 349–358.

Kulikov, S., Chirkov, S., Il'ina, A., Lopatin, S., & Varlamov, V. (2006). Effect of the molecular weight of chitosan on its antiviral activity in plants. *Applied Biochemistry and Microbiology*, *42*, 200–203.

Langlet, J., Gaboriaud, F., & Gantzer, C. (2007). Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. *Journal of Applied Microbiology*, *103*, 1632–1638.

Leuba, S., & Stossel, P. (1986). Chitosan and other polyamines: antifungal activity and interaction with biological membranes. In R. A. A. Muzzarelli, C. Jeuniaux, & C. Gooday (Eds.), *Chitin in nature and technology* (pp. 215–217). New York, NY: Plenum Press.

Li, J., Zivanovic, S., Davidson, P. M., & Kit, K. (2010). Characterization and comparison of chitosan/PVP and chitosan/PEO blend films. *Carbohydrate Polymers*, *79*, 786−791.

Li, J., Zivanovic, S., Davidson, P. M., & Kit, K. (2011). Production and characterization of thick, thin and ultra-thin chitosan/PEO films. *Carbohydrate Polymers*, *83*, 375−382.

Li, M., Chen, X., & Yanbin, X. (2009). The role of chitosan in protection of tomato from leaf mould caused by *Fulvia fulva* (cooke) ciffri. In J. Liu, S. Vittori, & C. Yang (Eds.), *International conference of national product and traditional medicine location: NW Univ, Xian, Peoples R China. Oct. 16−18, 2009: Vols 1−2. Proceedings of 2009 international conference of natural product and traditional medicine* (pp. 177−182).

Li, Q., Dunn, E. T., Grandmaison, E. W., & Goosen, M. F. A. (1997). Application and properties of chitosan. In M. F. A. Goosen (Ed.), *Applications of chitin and chitosan* (pp. 3−29). Lancaster, PA: Technomic Publishing Co., Inc.

Lin, S., Mao, S., Guan, Y., Luo, L., Luo, L., & Pan, Y. (2012). Effects of dietary chitosan oligosaccharides and *Bacullus coagulans* on the growth, innate immunity and resistance of koi *(Cyprinus carpio koi)*. *Aquaculture*, *342−343*, 36−41.

Lin, S. B., Lin, Y. C., & Chen, H. H. (2009). Low molecular weight chitosan prepared with the aid of cellulose, lysozyme and chitinase: characterization and antimicrobial activity. *Food Chemistry*, *116*, 47−53.

Liu, H., Du, Y. M., Wang, X. H., & Sun, L. P. (2004). Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology*, *95*, 147−155.

Liu, J., Tian, S., Meng, X., & Xu, Y. (2007). Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. *Postharvest Biology and Technology*, *44*, 300−306.

Liu, N., Chen, X. G., Park, H. J., Liu, C. G., Liu, C. S., Meng, X. H., et al. (2006). Effect ofMW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymers*, *64*, 60−65.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z., & Yao, K. D. (2001). Antibacterial action of chitosan and carboxymethylated chitosan. *Journal of Applied Polymer Science*, *79*, 1324−1335.

Lytle, D., Rice, E. W., Johnson, C. H., & Fox, K. R. (1999). Electrophoretic mobilities of *Escherichia coli* O157:H7 and wild-type *Escherichia coli* strains. *Applied and Environmental Microbiology*, *65*(7), 3222−3225.

Maezaki, Y., Tsuji, K., Kawai, Y., Akimoto, M., Tsugita, T., Takeeawa, W., et al. (1993). Hypocholesterolemic effect of chitosan in adult males. *Bioscience, Biotechnology and Biochemistry*, *57*, 1439−1444.

Massouda, D. F., Visioli, D., Green, D. A., & Joerger, R. D. (2012). Extruded blends of chitosan and ethylene copolymers for antimicrobial packaging. *Packaging Technology and Science*, *25*(6), 321−327.

Mellegard, H., From, C., Christensen, B., & Granum, P. E. (2011). Inhibition of Bacillus cereus spore outgrowth and multiplication by chitosan. *International Journal of Food Microbiology*, *149*, 218−225.

Meng, X., Yang, L., Kennedy, J. F., & Tian, S. (2010). Effects of chitosan and oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit. *Carbohydrate Polymers*, *81*, 70−75.

Michael, L. M. (2005). *Significance of cell surface charge on microbial susceptibility to chitosan*. (MS thesis, University of Tennessee).

Michael, L. M., Rajpal, G., Golden, D., & Zivanovic, S. (2005). *Flocculation of yeast cells as affected by natural biopolymer chitosan*. Institute of Food Technologists Annual Meeting; July 16−20, 2005; New Orleans, LA. Abstract 89D-22.

Michael, L. M., Zivanovic, S., & Golden, D. A. (2004). *Surface charge of microbial cells and biopolymers as affected by environmental conditions*. Institute of Food Technologists Annual Meeting; July 12−16, 2004; Las Vegas, NE. Abstract 99D-25.

Muzzarelli, R. A. A. (1977). *Chitin*. Oxford, UK: Pergamon Press.

Muzzarelli, R. A. A. (2010). Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine Drugs*, *8*, 292−312.

Neyrinck, A. M., Bindels, L. B., De Backer, F., Pachikian, B. D., Cani, P. D., & Delzenne, N. M. (2009). Dietary supplementation with chitosan derived from mushrooms changes adipocytokine profile in diet-induced obese mice, a phenomenon linked to its lipid-lowering action. *International Immunopharmacology*, *9*(6), 767−773.

No, H. K., Kim, S. H., Lee, S. H., Park, N. Y., & Prinyawiwatkul, W. (2006). Stability and antimicrobial activity of chitosan solutions affected by storage temperature and time. *Carbohydrate Polymers*, *65*, 174−178.

No, H. K., Park, N. Y., Lee, H. J., Hwang, H. J., & Meyers, S. P. (2002). Antimicrobial activities of chitosans and chitosan oligomers with different molecular weights on spoilage bacteria isolated from tofu. *Journal of Food Science*, *67*(4), 1511−1514.

No, H. K., Park, Y., Lee, H., & Meyers, S. P. (2002). Antimicrobial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65−72.

No, H. K., Meyers, S. P., Prinyawiwatkul, W., & Xu, Z. (2007). Applications of chitosan for improvement of quality and shelf life of foods: a review. *Journal of Food Science*, *72*, 65−72.

Okamoto, Y., Tomita, T., Minami, S., Matsuhashi, A., Tanioka, S., & Shigemasu, S. (1995). Effects of chitosan on experimental abscess with *Staphylococcus aureus* in dogs. *Journal of Veterinary Medical Science*, *57*, 765−767.

Omura, Y., Renbutsu, E., Morimoto, M., Salmoto, H., & Shigemasa, Y. (2003). Synthesis of new chitosan derivatives and combination with biodegradable polymer. *Polymers and Advanced Technologies*, *14*(1), 35−39.

Pa, J. H., & Yu, T. L. (2001). Light scattering study of chitosan in acetic acid aqueous solutions. *Macromolecular Chemistry and Physics*, *202*, 985−991.

Papineau, A. M., Hoover, D. G., Knorr, D., & Farkas, D. F. (1991). Antimicrobial effect of water-soluble chitosans with high hydrostatic pressure. *Food Biotechnology*, *5*, 45−57.

Peter, M. G. (1995). Applications and environmental aspects of chitin and chitosan. *Journal of Macromol Science: Pure and Applied Chemistry*, *A32*, 629−640.

Poelstra, K. A., van der Mei, H. C., Gottenbos, B., Grainger, D. W., van Horn, D. W., van Horn, J. R., et al. (2000). Pooled human immunoglobulins reduce adhesion of *Pseudomonas aeruginosa* in a parallel plate flow chamber. *Journal of Biomedical Materials Research*, *51*, 224−232.

Rauw, F., Gardin, Y., Palya, V., Anbari, S., Gonze, M., Lemaire, S., et al. (2010). The positive adjuvant effect of chitosan on antigen-specific cell-mediated immunity after chickens vaccination with live Newcastle disease vaccine. *Veterinary Immunology and Immunopathology*, *134*, 249−258.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*, 1−27.

Reddy, M. V. B., Arul, J., Angers, P., & Couture, L. (1999). Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearum* and improves seed quality. *Journal of Agricultural and Food Chemistry*, *47*, 1208−1216.

Reddy, M. V. B., Belkacemi, K., Corcuff, R., Castaigne, F., & Arul, J. (2000). Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit. *Postharvest Biology and Technology*, *20*, 39−51.

Rhoades, J., & Roller, S. (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Applied and Environmental Microbiology*, *66*(1), 80–86.

Rijnaarts, H. H. M., Norbe, W., Lyklema, J., & Zehnder, A. J. B. (1995). The isoelectric point of bacteria as an indicator for the presence of cell surface polymers that inhibit adhesion. *Colloids and Surfaces B: Biointerfaces*, *4*, 191–197.

Rinaudo, M., Milas, M., & Dung, P. L. (1993). Characterization of chitosan. Influence of ionic strength and degree of acetylation on chitin expansion. *International Journal of Biological Macromolecules*, *15*, 281–285.

Rivero, S., Garcia, M. A., & Pinotti, A. (2010). Crosslinking capacity of tannic acid in plasticized chitosan films. *Carbohydrate Polymers*, *82*(2), 270–276.

Roberts, G. A. F. (1992). *Chitin chemistry*. London: Macmillan Press, Ltd.

Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*, *47*, 67–77.

Roller, S., & Covill, N. (2000). The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimps salads. *Journal of Food Protection*, *63*(2), 202–209.

Sankararamakrishnan, N., Dixit, A., Iyengar, L., & Sanghi, R. (2006). Removal of hexavalent chromium using a novel cross linked xanthated chitosan. *Bioresource Technology*, *97*, 2377–2382.

Sashiwa, H., & Aiba, S. (2004). Chemically modified chitin and chitosan as biomaterials. *Progress in Polymer Science*, *29*, 887–908.

Schreiber, S., Zivanovic, S., Bozell, J., & Hayes, D. (2013). Introduction of primary antioxidant activity to chitosan for application as a multifunctional food additive. *Food Hydrocolloids*, *33*(2), 207–214.

Seyfarth, F., Schliemann, S., Elsner, P., & Hipler, U. C. (2008). Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-D-glucosamine against *Candida albicans, Candida krusei* and *Candida glabrata*. *International Journal of Pharmaceutics*, *353*, 139–148.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y.-J. (1999). Food applications of chitin and chitosans. *Trends in Food Science & Technology*, *10*, 37–51.

Singh, T., Vesentini, D., Singh, A. P., & Daniel, G. (2008). Effect of chitosan on physiological, morphological, and ultrastructural characteristics of wood-degrading fungi. *International Biodeterioration & Biodegradation*, *62*, 116–124.

Skjak-Braek, G., Anthonsen, T., & Sandford, P. A. (1989). *Chitin and chitosan: Sources, chemistry, biochemistry, physical properties, and applications*. New York, NY: Elsevier Applied Science, 443 pp.

Stossel, P., & Leuba, J. L. (1984). Effect of chitosan, chitin, and some aminosugars on growth of various soil-borne phytopathogenic fungi. *Phytopatholgy Zeitschrift*, *111*, 82–90.

Strand, S. P., Varum, K. M., & Ostgaard, K. (2003). Interactions between chitosans and bacterial suspensions: adsorption and flocculation. *Colloids and Surfaces B: Biointerfaces*, *27*(1), 71–81.

Su, X. W., Zivanovic, S., & D'Souza, D. H. (2009). Effect of chitosan on the infectivity of murine norovirus, feline calicivirus, and bacteriophage MS2. *Journal of Food Protection*, *72*, 2623–2628.

Sudarshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology*, *6*, 257–272.

Sunikumar, M., Francis, T., Thachil, E. T., & Sujith, A. (2012). Low density polyethylene-chitosan composites: a study based on biodegradation. *Chemical Engineering Journal*, *204*, 114–124.

Tanaka, Y., Tanioka, S., Tanaka, M., Tanigawa, T., Kitamura, Y., Minami, S., et al. (1997). Effects of chitin and chitosan particles on BALB/c mice by oral and parenteral administration. *Biomaterials*, *18*, 591−595.

Tao, Y., Qian, L. H., & Xie, J. (2011). Effect of chitosan on membrane permeability and cell morphology of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Carbohydrate Polymers*, *86*, 969−974.

Tsai, G. J., & Hwang, S. P. (2004). *In vitro* and *in vivo* antibacterial activity of shrimp chitosan against some intestinal bacteria. *Fisheries Science*, *70*, 675−681.

Tsai, G. J., & Su, W. H. (1999). Antibacterial activity of shrimp chitosan against *Escherichia coli*. *Journal of Food Protection*, *62*(3), 239−243.

Tsai, G. J., Wu, Z. Y., & Su, W. H. (2000). Antibacterial activity of chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. *Journal of Food Protection*, *63*(6), 747−752.

Uchida, Y., Izume, M., & Ohtakara, A. (1989). Preparation of chitosan oligomers with purified chitosanase and its application. In G. Skjak-Braek, T. Anthonsen, & P. Sandford (Eds.), *Chitin and chitosan: Sources, chemistry, biochemistry, physical properties and applications* (pp. 373−382). London: Elsevier Appl Sci.

Uhrich, K. E., Cannizzaro, S. M., Langer, R. S., & Shakesheff, K. M. (1999). Polymeric systems for controlled drug release. *Chemical Reviews*, *99*, 3181.

Van der Lubben, I. M., Verhoef, J. C., Borchard, G., & Junginger, H. E. (2001). Chitosan for mucosal vaccination. *Advanced Drug Delivery Reviews*, *52*, 139−144.

Vander, P., Varum, K. M., Domard, A., El Gueddari, N. E., & Moerschbacher, B. M. (1998). Comparison of the ability of partially *N*-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. *Plant Physiology*, *118*(4), 1353−1359.

Variainen, J., Ratto, M., Lantto, R., Nattinen, K., & Hurme, E. (2008). Tyrosinase-catalysed grafting of food-grade gallates to chitosan: surface properties of novel functional coatings. *Packaging Technology and Science*, *21*(6), 317−328.

Wang, G. H. (1992). Inhibition and inactivation of 5 species of foodborne pathogens by chitosan. *Journal of Food Protection*, *55*, 916−919.

Weidenmaier, C., & Peschel, A. (2008). Teichoic acid and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nature Reviews Microbiology*, *6*, 276−287.

Wu, T., Zivanovic, S., Draughon, F. A., Conway, W. S., & Sams, C. E. (2005). Physicochemical properties and bioactivity of fungal chitin and chitosan. *Journal of Agricultural and Food Chemistry*, *53*(10), 3888−3894.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters*, *11*, 1699−1701.

Young, D. H., & Kauss, H. (1983). Release of calcium from suspension-cultured *Glycine max* cells by chitosan, other polycationics, and polyamines in relation to effects on membrane permeability. *Plant Physiology*, *73*, 698−702.

Zhang, H. Z., He, Z. C., Liu, G. H., & Qia, Y. Z. (2009). Properties of different chitosan/low-density polyethylene antibacterial plastics. *Journal of Applied Polymer Science*, *113*(3), 2018−2021.

Zheng, L. Y., & Zhu, J. F. (2003). Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers*, *54*, 527−530.

Zivanovic, S., Basurto, C. C., Chi, S., Davidson, P. M., & Weiss, J. (2004). Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. *Journal of Food Protection*, *67*, 952−959.

Zivanovic, S., Li, J., Davidson, P. M., & Kit, K. (2007). Physical, mechanical and antimicrobial properties of chitosan/PEO blend films. *Biomacromolecules*, *8*(5), 1505−1510.

Evaluating natural antimicrobials for use in food products

9

D.O. Ukuku, S. Mukhopadhyay, V. Juneja, K. Rajkowski
U.S. Department of Agriculture, Wyndmoor, PA, USA

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9.1 Introduction

An antimicrobial agent can be defined as any compound, natural or synthetic, that can slow/inhibit microbial growth and/or kill and reduce microbial populations when used in food processing. The agent may induce bacterial injury that leads to recovery or death/inactivation depending on the food composition and storage conditions. Antimicrobial agents may be classified as chemical and biological agents, including plant- and vegetable-associated products found in nature. Also included among these groups are bacterial metabolites (Shelef & Seiter, 1993): by-products formed during metabolism. Bacterial metabolites are usually classified into primary and secondary metabolites. An example of primary metabolites used in food production is citric acid produced by *Aspergillus niger*. Secondary metabolites are usually organic compounds produced during modification of primary metabolite syntheses. Examples of secondary metabolites include but are not limited to antibiotics such as erythromycin and bacitracin, nisin, and atropine, derived from various plants.

9.2 The advantages of using antimicrobials in food preservation

Thermal processing is widely used by the food industry to inactivate food-borne pathogens during food processing. However, thermal processing has been shown to impair the sensory/flavor characteristics of the treated food (Mazzotta, 2001). For heat-sensitive foods such as fruits and vegetables, thermal treatment is not advisable (Ukuku, 2004; Ukuku, Bari, Kawamoto, & Isshiki, 2005). The U.S. Food and Drug Administration (FDA) stipulated and mandated the 5-log pathogen-reduction performance standard, which food processors should follow and strive to achieve (FDA, 2001). The 5-log pathogen-reduction performance standard required by the regulation means that any processing treatments used by the food industry must achieve at least a

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100,000-fold decrease in the number of specific human pathogenic bacteria or microorganisms. Therefore, there is a need for alternative processing treatments that can achieve a 5-log reduction in food-borne pathogens (Mazzotta, 2001; Sizer & Balasubramaniam, 1999) without causing adverse effects on the sensory/flavor characteristics. Consumers are becoming more health conscious and tend to opt for less heat-processed foods. As a result, food-processing industries are responding by introducing minimally processed foods in the supermarkets.

Contamination of foods with human bacterial pathogens and the pathogens' ability to survive in low- to high-acid foods has caused numerous illnesses, some of which have proven to be fatal (Bock, 2008; CDC, 1991, 1996a, 1996b, 1997, 1999; Cody et al., 1999; Doyle, 1991; Griffin, 1993; Salmon et al., 1989; Ukuku, Geveke, Zhang, & Cooke, 2008). For example, consumption of contaminated fruit juices was implicated in at least 28 food-borne illness outbreaks (Cody et al., 1999). Eleven of these 28 outbreaks (almost 40%) were associated with *Salmonella* spp. and eight of 28 (close to 30%) were caused by *Escherichia coli* O157:H7. The microflora of foods is of practical significance to producers, processors, and consumers (Ukuku & Sapers, 2001, 2007). Multistate outbreaks of food-borne disease (occurring in the years 2000, 2001, and 2002) were due to *Salmonella* Poona (CDC, 2002). In all the outbreaks noted so far, reports mentioned melons, which had been precut and held at unknown temperatures for some period of time at retail prior to being purchased and consumed (Ukuku, Mukhopadhyay, & Olanya, 2013; Ukuku, Olanya, Geveke, & Sommers, 2012).

The use of antimicrobials as a processing aid for heat-sensitive foods will eliminate or reduce the incidence of these outbreaks involving *E. coli* O157:H7 and *Salmonella* and the concern about the safety of consuming minimally processed heat-sensitive food. The specificity for the use of antimicrobials and its efficacy are dependent on the type of food. For example, the suggested levels for sorbic acid or potassium sorbate in dried fruits, wine, and processed fish are 0.02−0.05%, 0.01−0.04%, and 0.05−0.10%, respectively. For sodium or potassium benzoate, the suggested levels in fruit drinks, fruit salads, and jam and jelly are 0.05−0.1%, 0.075−0.10%, and 0.10%, respectively. Among the major advantages of using antimicrobials in heat-sensitive foods is that they keep the food closer to the natural or fresh state compared to thermal treatments, which also affect the flavor characteristics.

9.3 The use of natural antimicrobials in food preservation

Natural antimicrobials are mostly short-chain organic acid compounds used in food preparation to extend the shelf life of foods. Some are made up of specific amino acids and are called proteins. Short-chain organic acid compounds or substances are generally regarded as safe and in most cases are called GRAS compounds (FDA, 2014). Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act, a food additive substance is defined as anything that is intentionally added to food and

such substance is subject to premarket review and approval by the FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use or unless the use of the substance is otherwise excluded from the definition of a food additive or substance used in food before 1958, through experience based on common use in food.

Chemical preservatives are usually classified into two groups: antimicrobial agents and antioxidants. However, antimicrobial agents are substances that can destroy microbial populations in food. These substances include but are not limited to organic acids and their salts. Examples of organic acids and their salts used in the food system are acetic acids, which are classified as sodium acetate, potassium acetate, calcium acetate, and sodium diacetate. They are mostly used for acidification and sequestering of metals in foods and to enhance food flavor. Their primary targets are yeast and bacteria but are less effective against mold. They are mostly used for making breads and other baked goods, pickled meats and fish products, catsup, mayonnaise, and pickles. Benzoic acid derived from fruits such as cranberries, raspberries, prunes, cloves, and cinnamon is the oldest and most commonly used antimicrobial agent.

Sulfites, nitrites, and epoxide are other agents used in the food system to achieve a specific goal. Sulfites are also considered antioxidants and can be used to inhibit the browning reaction. Nitrite is bacteriostatic and is mostly used to control *Clostridium botulinum*. It also stabilizes the pink color of meat and improves flavor. Epoxides are classified into ethylene oxide and propylene oxide. They are heat labile and therefore are mostly used for sterilization. They are used in ground spices and low-moisture products and are effective against yeast, spores, and mold. The recommended level is $\leq$50 ppm.

Antimicrobial activity of natural plant extracts such as quercetin, thymoquinine, xanthohumol, chlorogenic acid, and myricetin has been reported (Certin-Karaca, 2011).

Other compounds in this category employed as food preservatives include, but are not limited to, sodium propionate, sodium lactate, and potassium sorbate, and are salts of weak acids. These compounds are found in nature and are produced by certain bacteria. For example, nisin, a bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* (Gross & Morell, 1971; Jung, 1991a, 1991b; Ray, 1992; Shiba et al., 1991), has a broad spectrum of inhibitory activity against Gram-positive vegetative bacteria and spore formers (Hurst & Hoover, 1993; Stevens, Klapes, Sheldon, & Klaenhammer, 1992a, 1992b; Stevens, Sheldon, Klapes, & Klaenhammer, 1991; Ukuku & Shelef, 1996, 1997). There are several reports that nisin used in combination with a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), exhibits a bactericidal effect toward both Gram-positive and gram-negative bacteria (Cutter & Siragusa, 1995; Stevens et al., 1991, 1992a, 1992b; Ukuku & Fett, 2002; Ukuku, Pilizota, & Sapers, 2004; Ukuku et al., 2013). Several researchers have suggested a combination of antimicrobials for inactivation of bacteria in foods (Bari et al., 2005; Gywali, Ibrahim, Hasfa, Smqadri, & Haik, 2011; Huang & Chen, 2011; Ibrahim, Yang, & Seo, 2008; Ukuku & Shelef, 1994, 1997; Ukuku et al., 2013). Another natural antimicrobial compound from a biological system (phage) has been reported to inactivate bacteria in foods. Bacteriophage/phage are obligate

intracellular parasites that replicate inside bacteria. The nucleic acid in phage can be either DNA or RNA, but not both, and their size varies depending upon the phage. The use of these biological compounds for inactivation of bacteria in food is known as biocontrol but has not been widely accepted.

9.4 Combining antimicrobials with other preservation techniques

In general, the term antimicrobials refers to both thermal and nonthermal processing treatments used to improve the shelf-life of the treated food. In this chapter, only the nonthermal chemical GRAS antimicrobials are discussed. Chlorination of wash water has been reported to prevent microbial contamination in produce processing lines (Virto, Manas, Alvarez, Condon, & Raso, 2005; Yildiz, 1994). However, chlorination may result in the formation of potentially carcinogenic chlorinated organic compounds (Wei, Cook, & Kirk, 1985); therefore there is much interest in developing safer alternative antimicrobials/sanitizers. Bari et al. (2005) reported a combined efficacy of nisin or pediocin with individual concentrations of sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population in inoculated fresh-cut produce. Other chemical antimicrobials, such as hydrogen peroxide (H_2O_2; CFR, 2013) treatment for sanitizing fruit surfaces before making fresh-cut preparations, have been proposed as substitutes for chlorinated compounds (Sapers, Miller, & Mattrazo, 1999; Sapers & Simmons, 1998; Sapers, Walker, Sites, Annous, & Eblen, 2003; Ukuku, 2004; Ukuku et al., 2005, 2012). The efficacy of hydrogen peroxide at all concentrations tested was better at reducing *Salmonella* populations on honeydew rind surface than on cantaloupe (Table 9.1). The authors concluded that the observed differences in bacterial inactivation by hydrogen were due to the surface structures of the two melons tested.

Sodium chloride salt (NaCl) has been used historically as an antimicrobial, mainly to prevent food spoilage by microorganisms, through the reduction of water activity to a point at which bacteria are incapable of growing. Another novel inhibitory activity of sodium chloride depends on its ability to dissociate when it comes in contact with the food liquid. Dissociation of this salt is dependent on the pH of the food. It was determined that the dissociation can be completed using electrolysis on a solution of sodium chloride, which results in an acidic and a basic component. One such system is now available that can produce a continuous supply of the acidic (anolyte) and basic (catolyte) components. Anolyte's main ingredient (92%) is electrolytically generated hypochlorous acid and is approved as a sanitizer for food contact surfaces by the U.S. FDA and U.S. Environmental Protection Agency (EPA) (CFR, 2009, 2011, 2012). Anolyte, used as a bactericidal and fungicidal sanitizer for food contact surfaces, is nontoxic, is environmentally friendly (noncorrosive), and leaves no residue (requires no rinsing) and is considered a "green biocide" (Robinson, Thorn, & Reynolds, 2013).

The choice of antimicrobials in food preparation is limited to those that do not adversely affect the sensory characteristics of the food, and this restriction may pose

Table 9.1 Efficacy of hydrogen peroxide at reducing *Salmonella* populations: honeydew rind surface compared to cantaloupe

		Salmonella population[a]			
Melon	**Treatment**	**Log CFU/ cm^2 whole melon**	**Log reduction**	**Log CFU/g fresh-cut pieces**	**Log reduction**
Cantaloupe	Control	4.41 ± 0.12	–	2.12 ± 0.14	–
	Water	4.28 ± 0.16	0.13	2.06 ± 0.14	0.06
	H_2O_2 (2.5%)	1.89 ± 0.04	2.56	0.39 ± 0.09	1.73
	H_2O_2 (5%)	2.12 ± 0.12	2.29	0.26 ± 0.09	1.86
Honeydew	Control	3.13 ± 0.14		1.32 ± 0.13	–
	Water	2.67 ± 0.16	0.51	1.25 ± 0.10	0.07
	H_2O_2 (2.5%)	ND	~3.00	ND	~1.32
	H_2O_2 (5%)	ND	~3.00	ND	~1.32

(–) means sample was negative for the pathogen even after enrichment procedures. ND = Pathogen was not determined from the sample after treatment.
[a]Values are mean ± SD of duplicate determinations from three trial experiments.

difficult challenges to food manufacturers or researchers. The most acceptable antimicrobials in food preparation are those that do not have an impact on the flavor or appearance of the food. In certain food preparations in which specific sensorial characteristics of the food may be impaired, it is advisable to combine antimicrobials with other treatments (hurdle technology) during processing. Ukuku et al. (2004) reported that fresh-cut cantaloupes and honeydew melons treated with 1% H_2O_2 solution had better appearance rating and overall acceptability rating compared to controls and those exposed to water alone for the same amount of time Figure 9.1(a) and (b).

The hurdle technology is a process in which more than one technique is employed to kill bacteria in food during processing to achieve a microbial-safe food. For example, a lower concentration of antimicrobials may be used, followed by nonthermal or minimal thermal treatments. Ukuku and Geveke (2010) reported the use of ultraviolet (UV) light and radiofrequency (RF) electric fields for inactivation of bacteria in apple juice. Other researchers have documented the use of nonthermal technologies in combination with antimicrobials for reducing the populations of *Salmonella* spp., *E. coli* O157:H7, and *Li. monocytogenes* in food (Gyawali et al., 2011; Huang & Chen, 2011; Ibrahim, et al., 2008; Mukhopadhyay, Ukuku, Fan, & Juneja, 2013; Quintero-Ramos, Churey, Hartman, Barnard, & Worobo, 2004). Several antimicrobial agents combined with thermal and nonthermal technologies for food processing have been commercialized (FDA, 2002). However, some of these processing technologies, especially nonthermal

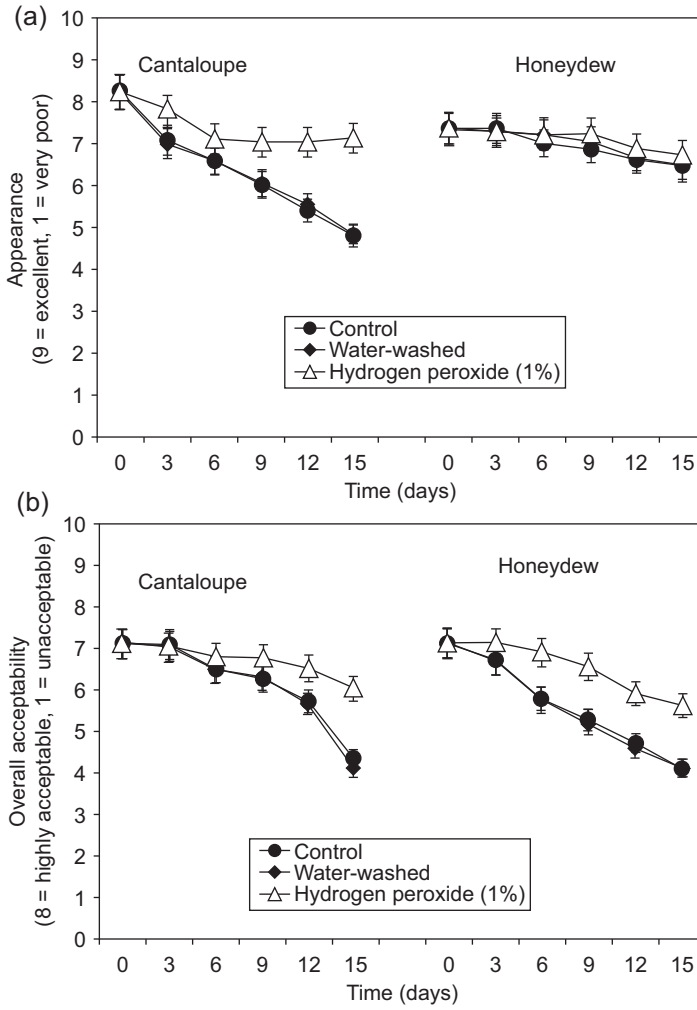

Figure 9.1 (a) Appearance rating for fresh-cut pieces from treated and untreated whole melons stored at 10 °C. Values represent means ± standard deviation of values from three separate experiments. *(Source: Ukuku (2004), with permission.)* (b) Overall acceptability of fresh-cut pieces from melons washed in 1% hydrogen peroxide and stored at 5 °C. Values represent means ± SE of values from three separate experiments. *(Source: Ukuku, 2004.)*

processes such as biological controls, have not been fully explored by the food industry (Olanya, Ukuku, Annous, Niemira, & Sommers, 2013). Also, the use of several UV apparatuses for inactivation of bacteria in food has been reported (Basaran, Quintero-Ramos, Moake, Churey, & Worobo, 2004; Duffy, Churey, Worobo, & Schaffner, 2000; Hanes et al., 2002; Koutchma, Keller, Chirtel, & Parisi, 2004; Koutchma & Parisi, 2004; Mukhopadhyay et al., 2013; Shama, 1999; Ukuku et al., 2008; Woo, Rhee, & Park, 2000; Wright, Sumner, Hackney, Pierson, & Zoecklein,

2000). Some of these antimicrobials can be referred to as food preservatives, which include antioxidants and antibrowning agents. Because so much information about bacterial inactivation in food using heat has been reported and is mostly understood, the emphasis here is mostly on natural antimicrobial agents.

Sodium propionate, sodium lactate, potassium sorbate, and sodium benzoate are salts of weak organic acids employed as food preservatives. An investigation of the sensitivity of *Li. monocytogenes* to sodium propionate showed that >0.2% was inhibitory in broth at pH 5.0 (Eklund, 1985, 1989; Woolford & Anderson, 1945). A concentration of 0.3% was effective in cold-pack cheese foods when used in combination with acetic acid (Ryser & Marth, 1988). Sodium, potassium, and calcium lactate (2−4%) have been shown to control the growth of aerobes and anaerobes in meats and to have antibotulinal and antilisterial activities (Bacus & Bontenbal, 1991; Mass, Glass, & Doyle, 1989; Papadopoulos et al., 1991a, 1991b; Shelef & Yang, 1991). The susceptibility of *Li. monocytogenes* to sorbic acid was reported by El-Shenawy and Marth (1989), and studies in pork liver sausage confirmed antilisterial effects of this acid and, to a lesser degree, of the potassium salt (Hu & Shelef, 1996; Perumalla et al., 2012).

Several natural antimicrobials of plant origin were shown to be inhibitory to pathogenic and spoilage microorganisms (Certin-Karaca, 2011). The author concluded that the chemical structure of these plant-related antimicrobials plays a major role in their efficacy. For example, phenolic compounds extracted from vegetables, fruits, herbs, and spices were shown to be highly effective against the growth of pathogenic bacteria. However, the author reported variations in antimicrobial susceptibility of Gram-positive bacteria versus Gram-negative bacteria.

9.5 Factors affecting the biocidal activity of natural antimicrobials

Anolyte (i.e., HOCl) produced by electrolysis of a saturated NaCl solution with a resulting pH of 6.0−6.5 is classified as a weak acid and has a pK_a of ~7.5. Dissociation of this acid yields H^+ and OCl^-, a strong oxidizing agent that contains the most active form of residual chlorine (Dychdala, 2001; McDonnell & Russell, 1999; McKenna & Davies, 1988). The biocidal activity of available chlorine (AC) in solution is pH dependent; at pH 6 it took 2.5 min for the AC to inactivate bacterial spores by 99%, and increased contact time was required when the pH was increased (Dychdala, 2001). Rajkowski and Sommers (2012) used anolyte (300 ppm, pH 6.4, 3 min at 23 °C) to inactivate the background microflora, *Salmonella*, and *Li. monocytogenes* on catfish fillets. They reported a 1.0 $\log_{10}$ CFU/g reduction in the background microflora and *Salmonella* but no reduction in *Li. monocytogenes*.

Nutrient composition and food pH play a major role in the activities of antimicrobials. Slight changes in pH affect microbial physiology, which may lead to inhibition or death (Salmond, Kroll, & Booth, 1984). Changes in food pH alone can be used to control microbial growth in food. For example, the Adenosine triphosphate (ATP) level of bacterial cells grown at pH 5 without antimicrobial agent was one log cycle

less than those at pH 6 and 7 during 9 h of incubation (Ukuku & Shelef, 1994). The author reported that the mechanism of bacterial inactivation by propionate, lactate, and sorbate in nutrient broth did not involve changes in intracellular ATP of the bacteria. In a normal growing cell, the intracellular ATP level of bacteria will remain at equilibrium and can be used to estimate bacterial colony-forming units (Ukuku, 1995; Ukuku & Shelef, 1994, 1996). The minimum threshold for CFU/ml to ATP/ml was established at 5.0 $\log_{10}$ CFU/ml and above where the corresponding ATP level showed a positive correlation. Therefore, any perturbation of the ATP level by the use of antimicrobials in food processing will indicate subcellular interaction of the antimicrobial leading to bacterial inactivation.

Differences in the intracellular ATP of *Salmonella* spp., *E. coli* K-12, *E. coli* O157:H7, and *Li. monocytogenes* in nutrient broth at pH 7.2 in relation to population growth are shown in Figure 9.1. When the broth pH was adjusted to 5 and 6, similar observations on survival and growth population in relation to intracellular ATP were noted (Ukuku, 1995). In another study (Ukuku & Shelef, 1994), in which growth behavior and intracellular ATP of *Li. monocytogenes* in broth at pH 5 amended with 0.3% potassium sorbate (KS), 3% sodium lactate (NaL), or 2% sodium propionate (NaP) were investigated, the antimicrobials inhibited growth but did not affect intracellular ATP (Figures 9.2 and 9.3). The data suggest that the mechanism of inhibition of *Li. monocytogenes* by these antimicrobials does not involve depletion of intracellular ATP compared to nisin + EDTA (Okereke & Montville, 1992; Ukuku & Shelef, 1996). The antimicrobial activity of short-chain fatty acids is generally associated with their undissociated fraction. As to the specific mechanism of inhibition of each of these compounds, studies are limited. Buazzi and Marth (1992) reported inactivation of dehydrogenase in *Li. monocytogenes* exposed to propionate. Examination of lactates showed that the salts produced only a small reduction in water activity, which was insufficient to explain the inhibitory effect, and they did not lower the intracellular pH (Shelef, 1994). Several mechanisms of inhibition have been proposed for sorbate, including neutralization of the proton-motive force needed for substrate transport, inhibition of the electron transport system, synthesis or depletion of ATP, and

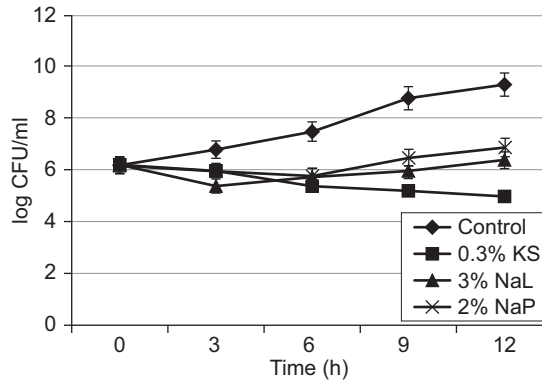

Figure 9.2 Effect of natural antimicrobial agents on colony-forming units of *Listeria monocytogenes* in tryptic soy broth at pH 5. Values are means of three studies with duplicate determinations ± SD.

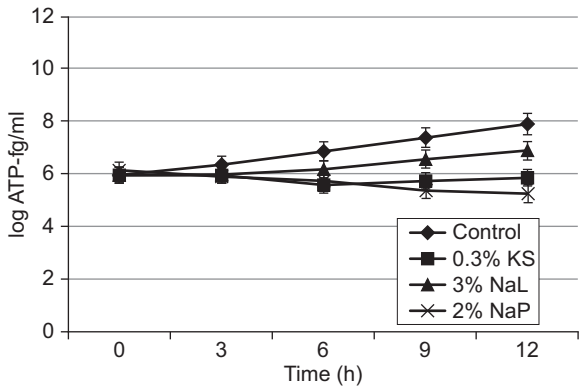

Figure 9.3 Effect of natural antimicrobial agents on intracellular ATP of *Listeria monocytogenes* in tryptic soy broth at pH 5. Values are means of three studies with duplicate determinations ± SD.

inhibition of transport enzymes or metabolic energy utilization by amino acid transport systems (Eklund, 1985; Salmond et al., 1984; Sofos, 1989).

Antimicrobial activities of short-chain organic acids used in foods are dependent on medium composition, type of microorganism, and water activity. For example, bacteria achieve optimum growth near or at neutral pH. Mold tolerates a broad pH range (2−10); however, pH < 6 favors mold growth over bacteria. Yeast tolerates a pH range of 3−10 but at pH 5 to 10, yeast will dominate over mold. Ukuku and Shelef (1997) reported inhibition of *Li. monocytogenes* by sodium lactate (0−4%), sodium propionate (0−2%), and potassium sorbate (0−1%) in nutrient broth at pH 5, 6, and 7 incubated at 35 °C. The authors also observed that the efficacy of these compounds increased when the pH of the nutrient broth decreased from 7 to 5, especially in 2% propionate, 4% lactate, and 1% sorbate. Ukuku and Shelef (1997) used the ATP technique to monitor antilisterial activity of specific individual short-chain organic acids including nisin, a bacteriocin, produced by *La. lactis* subsp. *lactis*. In this study, the authors reported that the mechanism of *Listeria* inhibition by the short-chain organic acids tested was different than that for nisin. Nisin caused membrane damage and leakage of intracellular ATP of bacteria, whereas sodium propionate, sodium lactate, and potassium sorbate did not. Similar membrane damage and leakage of intracellular ATP of *E. coli* bacteria in apple juice treated with RF alone was reported by Ukuku and Geveke (2010). In this study, the authors compared the efficacy of bacterial inactivation by RF and UV light alone and in combination and observed that the mechanisms were different (Figure 9.4). Gram-positive bacteria react differently to antimicrobials compared to Gram-negative bacteria because of differences in their structural membranes (Sung & Lee, 2008).

The mechanism for lactate inhibition could be through a different pathway because it does not reduce intracellular pH (Shelef, 1994). Lactate is a weak chelator of cations and it could be that the presence of lactate in the medium complexed some of the mineral ions such as Mg^{2+} available in the broth, preventing bacterial cells from metabolizing such ions. However, in nonnutrient broth such as phosphate-buffered saline (PBS) at pH 7, the surviving populations gradually declined in increasing

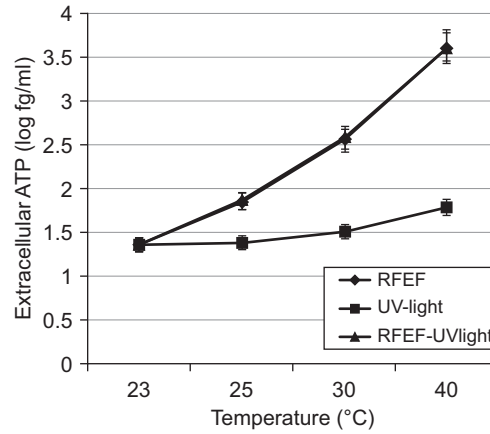

Figure 9.4 Leakage of intracellular ATP from UV light- and radiofrequency electric field-injured *Escherichia coli* cells into the medium. Values are means ± SD of duplicate determinations. *Source*: Ukuku and Geveke (2010), with permission.

concentrations of the sorbate salt at pH 5 and 6, but not in PBS amended with propionate and lactate, in which the bacterial cell numbers and intracellular ATP remained the same (Ukuku & Shelef, 1994). The authors attributed the observed behavior of bacteria to the buffering capacity of the medium, which resisted or delayed proton exchange when dissociation of salts occurred. The dissociation constants of the salts are pK_a 4.87 for propionate, 3.862 for lactate, and 4.75 for sorbate.

At pH 5, which is close to the dissociation constant of propionate and sorbate, more of these salts dissociate, resulting in proton influx with a subsequent change in the intracellular pH. It is likely that the perturbation of the intracellular pH may have reduced the cells' metabolism through diversion of intermediates or by decreasing metabolic activity. This effect may explain why in growing cells the intracellular ATP of cells exposed to propionate and sorbate was so low (Ukuku, 1995). Sheu and Freese (1972) reported a similar observation and explained that short-chain fatty acids function as antimicrobials by interfering with energy metabolism connected to the cell membrane. This implies that viability loss and the intracellular ATP depletion observed in the sorbate- and propionate-exposed cells at pH 5 could be due to intracellular pH acidification. Acidification of the intracellular pH by the dissociated salts could have denatured cellular enzymes, thereby inhibiting their activity in interconversion of specific metabolites needed for growth. This explanation is in agreement with results of Buazzi and Marth (1992), who studied the effect of propionate on *Li. monocytogenes* and concluded that inhibition did not involve cellular functions related to electron transport, cell wall, cell membrane, mRNA, or protein, but appeared to be associated with inhibition of synthesis or functioning of lactic dehydrogenase.

Most of the ions incorporated into nutrient broth are used by the reducing powers (NADH, FAD^+) inside the membrane to maintain biochemical processes in cells. However, compounds that interfere directly with the generation of either ATP or reducing powers will limit cell capacity to regulate intracellular pH (Booth & Kroll, 1989; Salmond et al., 1984). This may offer some explanation as to why the intracellular ATP of cells exposed to listeriostatic concentrations of lactate was reduced, but

remained higher than that of the propionate- or sorbate-exposed samples, which affects intracellular pH. The slight ATP increase observed in the lactate study (Ukuku, 1995; Ukuku & Shelef, 1994) confirmed the observation by Sheu, Salomon, Simmons, Sreeevalsan, and Freese (1975) that growth-inhibited cells were not necessarily ATP-depleted, and they attributed this effect to less inhibition of oxygen consumption than uptake of reducing substances.

The survival and growth behavior of aerobic mesophilic bacteria in apple cider amended with 0 or 300 IU/ml nisin in combination with 20 mM EDTA during storage at 5 °C for 14 days or 25 °C for 16 h are shown in Figure 9.5 (a) and (b). Bacterial populations in control and EDTA-containing samples increased, whereas the populations in nisin-treated samples had a longer lag phase before growth was observed, and the final populations were not as high as in the control or the EDTA samples. Populations of aerobic mesophilic bacteria of fresh-cut cantaloupe pieces dipped in nisin, EDTA, nisin + EDTA, lactate, nisin + lactate, sorbate, sorbate + lactate, or sorbate + lactate + nisin decreased during storage at 5 °C for 14 days (Figure 9.6). Similar decreases in populations of *Salmonella* spp. inoculated on fresh-cut cantaloupe pieces dipped in nisin solution amended with EDTA, lactate, sorbate, or nisin + lactate + sorbate combination stored at 5 °C for 14 days were observed (Figure 9.7). The antimicrobial activity of the nisin combination treatments was noted in all fresh-cut pieces, especially pieces dipped in lactate and nisin + EDTA, nisin + lactate, sorbate + lactate, and sorbate + lactate + nisin combinations. Ukuku and Fett (2004) reported similar findings on the reduction of *Salmonella* populations on inoculated whole cantaloupe rind dipped in nisin + EDTA for 5 min. The antimicrobial activity of nisin was specific at the bacteria membrane structure. Nisin was reported to affect the membrane potential of whole cells and cytoplasmic and artificial membrane vesicles (Rhur & Sahl, 1985).

Okereke and Montville (1992) reported that nisin dissipates the proton-motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. Another GRAS antimicrobial agent approved for use by the U.S. FDA for microbial inactivation, as already mentioned, is hydrogen peroxide (21 CFR184.1366). It is mostly used as a bleaching agent, antimicrobial agent, and oxidizing and reducing agent (Ukuku et al., 2004, 2013). Also, H_2O_2 is approved for preparations of milk treatment for cheese processing, modified whey, and thermophile-free starch. Populations of *Salmonella* spp. inoculated onto whole cantaloupe rind surfaces and the numbers transferred to fresh-cut pieces during fresh-cut preparation varied. Treatment of whole cantaloupe with up to 5% H_2O_2 before fresh-cut preparation reduced the transfer of *Salmonella* and the populations of transferred *Salmonella* were suppressed in fresh-cut pieces stored at 5 °C for 14 days (Figure 9.7). The antimicrobial specificity of H_2O_2 occurred at the DNA level (Imlay, Chin, & Linn, 1988; Imlay & Linn, 1986) and was reported to be concentration dependent (Ukuku et al., 2013).

Santiesteban-López et al. (2006) reported that the activity of antibacterial mixtures of potassium sorbate with a phenolic compound (thymol, carvacrol, or eugenol) against *E. coli*, *Listeria innocua*, *Salmonella* Typhimurium, and *Staphylococcus aureus* varied among the bacteria and was also dependent on the water activity (a_w) and the pH tested.

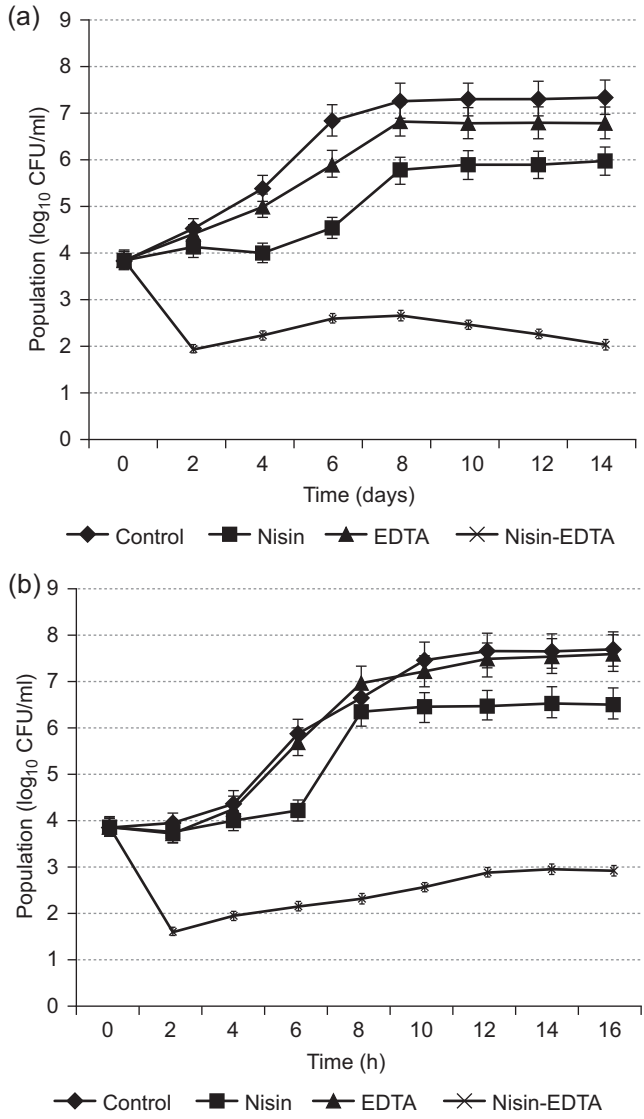

Figure 9.5 (a) and (b) Populations of mesophilic bacteria in apple cider containing nisin (0 and 300 IU/Ml) and ethylenediaminetetraacetic acid (EDTA) (20 mM) during storage at 5 °C for 14 days (a) and 25 °C for 16 h (b).
Source: Ukuku, Zhang, and Huang (2009), with permission.

In determining the efficacy of antimicrobial agents in foods, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) should be established. The MIC and MBC are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

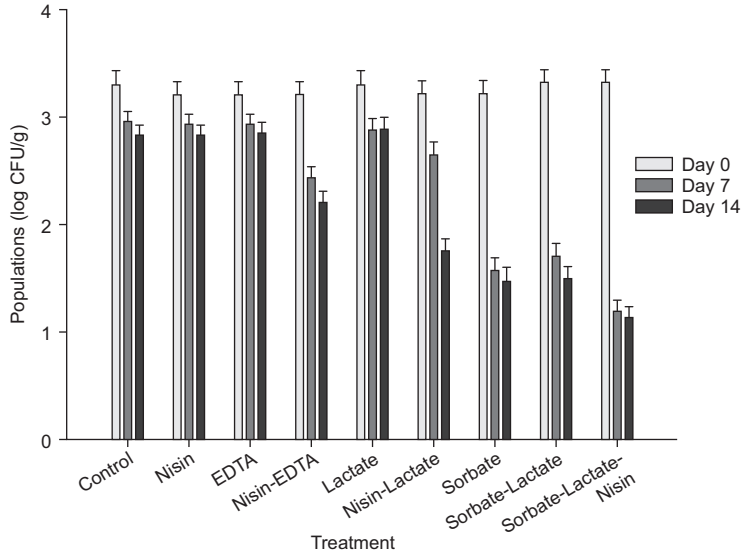

Figure 9.6 Survival and viability loss of aerobic mesophilic bacteria on fresh-cut cantaloupes treated with short-chain organic acids stored at 5 °C for 14 days. Values are means ± standard deviation for three experiments with duplicate determinations.
Source: Ukuku, Mukhopadhyay, and Olanya (2013), with permission.

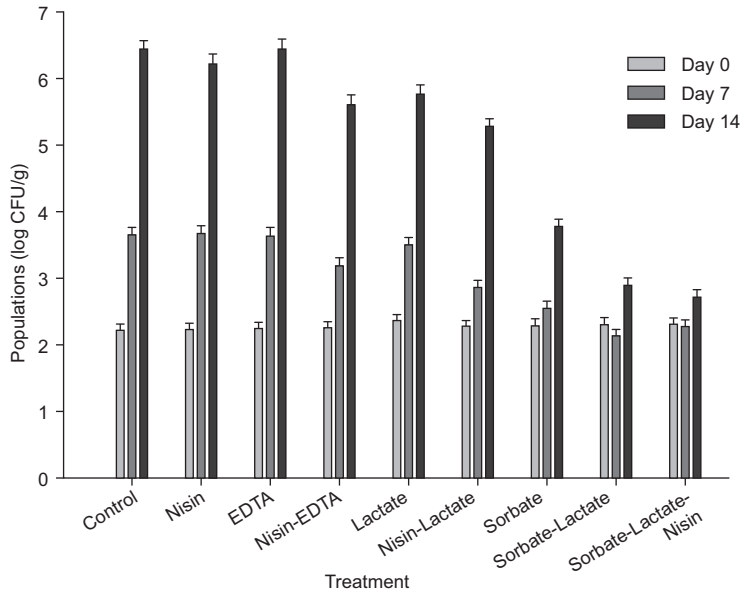

Figure 9.7 Survival and viability loss of *Salmonella* spp. on fresh-cut cantaloupes treated with short-chain organic acids stored at 5 °C for 14 days. Values are means ± standard deviation for three experiments with duplicate determinations.
Source: Ukuku, Mukhopadhyay et al. (2013), with permission.

incubation and the lowest concentration of an antimicrobial that will prevent the growth of an organism after subculture on antibiotic-free medium (Andrews, 2001). In that paper (Andrews, 2001), the author reported several ranges for MIC of specific antibiotics. Similarly, the author reported that in most cases, the disc diffusion method was used but there are other methods that can be used, especially where the obtained results show borderline values and appear to be inconclusive. Burt (2004) reported that most researchers cite MIC as a measure of antimicrobial performance of essential oils (EOs). The author goes on to list the most frequent terms in antimicrobial activity testing of EOs. In summary, Burt (2004) summarized and listed major components of selected EOs that exhibit antimicrobial properties and the MIC techniques used to test bacterial susceptibility, shown in Table 9.2. This is the standard used to evaluate the efficacy of bacterial susceptibility to antimicrobials.

9.6 The regulation of natural antimicrobials

The ability of pathogenic bacteria to adhere to food surfaces, including internalization, continues to be a potential food safety problem and a great concern to the food industries, regulatory agencies, and consumers. The presence of human bacterial pathogens in foods and outbreaks of diseases have led to costly recalls. For health reasons, consumers are demanding foods that are safe, fresher, more convenient, and inexpensive. Food industries and researchers are responding to this demand by designing and developing novel intervention technologies that keep food close to natural, especially heat-sensitive foods. To effectively control and minimize microbial contamination of food, the use of alternative control systems such as natural antimicrobials are required rather than thermal treatments. There were no food laws in the United States until 1906, when the Pure Food and Drug Act and the Meat Inspection Act were passed and the United States Department of Agriculture (USDA) was mandated to supervise the law (Ray & Buhnia, 2008). The law states that selling unwholesome, unsafe foods, including meat, in interstate commerce is illegal. There were no mandates by the federal government as to a specific standard for what, how, and how much chemical additives could be used in foods. Processed foods and other goods were easily transported across state lines and consumers were worried about the state of the goods. Therefore it was the responsibility of the government and the industry to uncover instances of food adulteration, and the regulation of these goods was under state law. The Meat Inspection Act required the USDA to inspect slaughterhouses and meat-processing facilities. The law also shifts the burden of proof that a substance added to meat is not at an unsafe level to the USDA. In 1938, the federal government passed the Food, Drug, and Cosmetic Act and gave the FDA the power to administer this law. Therefore, in the United States, the responsibilities of monitoring and regulating the origin, composition, quality, quantity, safety, labeling, packaging, and marketing of foods fall under several agencies. For antimicrobial use in food, the regulation authority falls under the USDA, FDA, Food Safety Inspection Service, and EPA.

Certain antimicrobial use falls under the jurisdiction of the FDA and the EPA. To clarify this jurisdiction, the FDA stated that antimicrobials used in or on food,

Table 9.2 MIC techniques used to test bacterial susceptibility to activity of essential oils (EOs)[a]

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool E-2-decanal	26% 20%	Delaquis, Stanich, Girard, and Mazza (2002)
Coriander	*C. sativum* (seeds)	Linalool E-2-decanal	70% –	Delaquis et al. (2002)
Cinnamon	*Cinnamomum-zeylandicum*	*Trans*-cinnamaldehyde	65%	Lens-Lisbonne, Cremieux, Maillard, and Balansard (1987)
Oregano	*Oreganum vulgare*	Carvacrol Thymol γ-Terpinene p-Cymene	Trace-80% Trace-64% 2–52% Trace-52%	Lawrence (1984), Prudent, Perineau, Bessiere, Michel, and Baccou (1995), Charai, Mosaddak, and Faid (1996), Sivropoulou, Papanikolaou, Nikolaou, and Kokkini (1996), Kokkini, Karousou, Dardioti, Krigas, and Lanaras (1997), Russo, Galletti, Bocchini, and Carnacini (1998), Daferera, Ziogas, and Polissiou (2000), Demetzos and Perdetzoglou (2001), and Marino, Bersani, and Comi (2001)

Continued

Table 9.2 Continued

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Rosemary	*Rosmarinus officinalis*	α-pinene Bomyl acetate Camphor 1,8-cineol	2–15% 0–17% 2–14% 3–89%	Daferera et al. (2000), Daferera, Ziogas, and Polissiou (2003), and Pintore et al. (2002)
Sage	*Salvia officinalis* L.	Camphor α-pinene β-pinene 1,8-Cineole α-tujone	6–15% 4–5% 2–10% 6–14% 20–42%	Marino et al. (2001)
Clove (bud)	*Syzygium aromaticum*	Eugenol Eugenyl acetate	75–85% 8–15%	Bauer, Garbe, and Surburg (2001)
Thyme	*Thymus vulgaris*	Thymol Carvacrol γ-Terpinene p-Cymene	10–65% 2–11% 2–31% 10–56%	Lens-Lisbonne et al. (1987), McGimpsey, Douglas, Van Klink, Beauregard, and Perry (1994), Cosentino et al. (1999), Marino, Bersani, and Comi (1999), Daferera et al. (2000), and Juliano, Mattana, and Usai (2000)

[a]EOs which have been shown to exert antibacterial properties *in vitro* or in food models and for which the composition could be found in the literature.
[b]Percentages of total volatiles rounded up to the nearest whole number.

including those used in or on edible food, in water that contacts edible food, and in the manufacture of, in or on, food-contact articles, subsequent to the enactment of the Food Quality Protection Act of 1996 (FQPA) and the Antimicrobial Regulation Technical Corrections Act of 1998 (ARTCA), are under their jurisdiction (FDA, 1999). The EPA regulates antimicrobials used in or on food packaging, such as slimicides used in the manufacture of food-contact paper, material preservatives used in the manufacture of food packaging and other food-contact articles, and those products intended to provide a sanitizing effect on food-contact surfaces. The FQPA is an amendment of the Federal Insecticide, Fungicide, and Rodenticide Act and the Federal Food, Drug, and Cosmetic Act (FFDCA), in which the definitions of "food additive" (section 201(s)) and "pesticide chemical" (section 201(q)) were changed. The FQPA became law on August 3, 1996.

The ARTCA was amended to reflect the changes in the definitions of "food additive" (section 201(s)) and "pesticide chemical" (section 201(q)) in the FFDCA as the regulatory authority for many antimicrobial products used in food-contact applications, as food additives previously, under section 409 of the FFDCA and FDA, were assigned to the EPA as "pesticide chemicals" under section 408 of the FFDCA. Antimicrobial use in food or food-contact surfaces and its jurisdiction is summarized in http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm077256.htm.

9.7 Conclusion

In conclusion, as changes in human health and the food industry continue to happen and new technologies are developed, certain accommodations to allow smooth operations between the industry and the regulatory agencies in the use of antimicrobials for enhancing food safety will improve. For example, the food additives amendment of 1958 that specifically classified food additives into two categories, direct additives and indirect additives, clearly improved the understanding for their use. The former refers to additives incorporated directly into the food supply and the latter to those used in various aspects of food-contact surfaces, such as packaging materials and processing machines. *E. coli* O157:H7, *Li. monocytogenes*, and *Salmonella* are all recognized food-borne pathogens (CDC, 1991; Doyle, 1991; Padhye & Doyle, 1992; Salmon et al., 1989). The presence of any one of these pathogens in food is a safety hazard for both consumers and the juice industries, as this has led to several food-borne disease outbreaks (CDC, 1996a, 1996b, 1999) and costly recalls. Therefore the need for alternative nonthermal processing treatments including the use of GRAS substances by the food industry will continue to grow. Similarly, knowledge of the levels of bacteria in food and understanding of the types of antimicrobials to use and the mechanisms by which they function should provide guidance to the food industry, researchers, and consumers alike in implementing hazard analysis and critical control point plans and good manufacturing practices (Francis & O'Beirne, 1998).

References

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*(Suppl. S1), 5−16.

Bacus, J., & Bontenbal, E. (1991). Controlling *Listeria*. *Meat and Poultry*, *37*, 64−65.

Bauer, K., Garbe, D., & Surburg, H. (2001). *Common fragrance and flavor materials: preparation, properties and uses* (p. 293). Weinheim: Wiley-VCH.

Bari, M. L., Ukuku, D. O., Kawasaki, T., Inatsu, Y., Isshiki, K., & Kawamoto, S. (2005). Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. *Journal of Food Protection*, *68*, 1381−1387.

Basaran, N., Quintero-Ramos, A., Moake, M. M., Churey, J. J., & Worobo, R. W. (2004). Influence of apple cultivars on inactivation of different strains of *Escherichia coli* O157:H7 in apple cider by UV irradiation. *Applied and Environmental Microbiology*, *70*, 6061−6065.

Bock, L. (2008). *State health lab used DNA fingerprints to find Listeria source*. http://www.telegram.com/apps/pbcs.dll/article?Date=2008217&Category+NEWS&ArtN Accessed 29.04.08.

Booth, I. R., & Kroll, R. G. (1989). The preservation of foods by low pH. In G. W. Gould (Ed.), *Mechanisms of action of food preservation procedures* (pp. 119−160). New York: Elsevier Appl. Sci.

Buazzi, M. M., & Marth, E. H. (1992). Sites of action by propionate on *Listeria monocytogenes*. *International Journal of Food Microbiology*, *15*, 109−119.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*, 223−253.

Centers for Disease Control (CDC). (1991). Multistate outbreak of *Salmonella* Poona infections − United States and Canada. *Morbidity Mortality Weekly Report*, *40*, 549−552.

Centers for Disease Control and Prevention (CDC). (1996a). Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Connecticut and New York, October 1996. *Morbidity Mortality Weekly Report*, *46*, 4−8.

Centers for Disease Control and Prevention (CDC). (1996b). Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple juice—British Columbia, California, Colorado, and Washington, October 1996. *Morbidity Mortality Weekly Report*, *45*(44), 975.

Centers for Disease Control and Prevention (CDC). (1997). Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Connecticut and New York, October 1996. *Morbidity Mortality Weekly Report*, *46*(1), 4−8.

Centers for Disease Control and Prevention (CDC). (1999). Outbreak of *Salmonella* serotype Muenchen infections associated with unpasteurized orange juice—United States and Canada, June 1999. *Morbidity Mortality Weekly Report*, *48*(27), 582−585.

Centers for Disease Control and Prevention (CDC). (2002). Multistate outbreaks of *Salmonella* serotype poona infections associated with eating cantaloupe from Mexico—United States and Canada, 2000−2002, November 22. *Morbidity Mortality Weekly Report*, *51*(46), 104−11064. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5146a2.htm. Accessed 14.08.14.

Certin-Karaca, H. (2011). *Evaluation of natural antimicrobial phenolic compounds against foodborne pathogens* (Master's theses). University of Kentucky. Paper 652. http://ukowledge.uky.edu/graschool_theses/652. Accessed 31.01.14.

Charai, M., Mosaddak, M., & Faid, M. (1996). Chemical composition and antimicrobial activities of two aromatic plants: origanum majorana L. and O. compactum Benth. *Journal of Essential Oil Research*, *8*, 657−664.

Code of Federal Regulations (CFR). (2009). *Food safety inspection service directive − safe and suitable ingredients*, 40 CFR 7120.1 Amended 20 10/05/09. www.fsis.usda.gov/OPPDE/rdad/FSISdirectives/7120.1amend14.pdf. Accessed 13.08.13

Code of Federal Regulations (CFR). (2011). *Food and drug administration approval of hypochlorous acid as a sanitizer*, 21 CFR 178.1010. www.gpo.gov/fdsys/pkg/CFR-2011-vol3/xml. Accessed 13.08.13

Code of Federal Regulations (CFR). (2012). *Environmental protection agency food-contact surface sanitizing solutions*, 40 CFR 180.940. www.gpo.gov/fdsys/pkg/CFR-2012-title40-vol25/xml/CFR-2012-title40-vol25-sec180-940.xml. Accessed 08.08.13.

Code of Federal Regulations (21CFR184.1366). (2013). Hydrogen peroxide. www.accessdata.fda.gov/.../CFRSearch.cf. Accessed 09.04.13.

Cody, S. H., Glynn, M. K., Farrar, J. A., Cairns, K. L., Griffin, P. M., Kobayashi, J., et al. (1999). An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Annals of Internal Medicine*, *130*, 202−209.

Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., & Palmas, F. (1999). In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Letters in Applied Microbiology*, *29*, 130−135.

Cutter, C. N., & Siragusa, G. R. (1995). Population reductions of gram negative pathogens following treatments with nisin and chelators under various conditions. *Journal of Food Protection*, *58*, 977−983.

Daferera, D. J., Ziogas, B. N., & Polissiou, M. G. (2000). GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on Penicillium digitatum. *Journal of Agricultural and Food Chemistry*, *48*, 2576−2581.

Daferera, D. J., Ziogas, B. N., & Polissiou, M. G. (2003). The effectiveness of plant essential oils in the growth of Botrytis cinerea, Fusarium sp. and Clavibacter michiganensis subsp. michiganensis. *Crop Protection*, *22*, 39−44.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*, *74*, 101−109.

Demetzos, C., & Perdetzoglou, D. K. (2001). Composition and antimicrobial studies of the oils of Origanum calcaratum Juss. and O. scabrum Boiss. et Heldr. from Greece. *Journal of Essential Oil Research*, *13*, 460−462.

Doyle, M. P. (1991). *Escherichia coli* O157:H7 and its significance in foods. *International Journal of Food Microbiology*, *12*, 289−302.

Duffy, S., Churey, J., Worobo, R. W., & Schaffner, D. W. (2000). Analysis and modeling of the variability associated with UV inactivation of *Escherichia coli* in apple cider. *Journal of Food Protection*, *63*, 1587−1590.

Dychdala, G. R. (2001). Chapter 7 − Chlorine and chlorine compounds. In S. S. Block (Ed.), *Disinfection, sterilization and preservation* (5th ed., pp. 79−157). Philadelphia, PA: Lippincott Williams & Wilkings.

Eklund, T. (1985). The effect of sorbic acid and esters of *p*-hydroxybenzoic acid on the proton motive force in *Escherichia coli* membrane vesicles. *Journal of General Microbiology*, *131*, 73−76.

Eklund, T. (1989). Organic acids and esters. In G. W. Gould (Ed.), *Mechanisms of action of food preservation procedures* (pp. 161−200). New York: Elsevier Appl. Sci.

El-Shenawy, M. A., & Marth, E. H. (1989). Behavior of *Listeria monocytogenes* in the presence of sodium propionate. *International Journal of Food Microbiology*, *8*, 85−94.

FDA. (1999). *Guidance for industry: Antimicrobial food additives*. A Nonbinding Recommendations. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm077256.htm. Accessed 10.02.14.

FDA. (2001). Guidance for industry: The juice HACCP regulation − Questions & answers final rule: Hazard Analysis and Critical Control Point (HAACP); Procedures for the safe and sanitary processing and importing of juice (Federal Register, November 9, 2001).

FDA. (2002). *Thermally-processed low-acid foods packaged in hermetically sealed containers*. Federal Register, 21CFR113. Rockville, MD: US Food and Drug Administration.

FDA. (2014). *Protecting and promoting your health, ingredients, packaging and labeling*. www.fda.gov/food/ingredientspackaginglabeling/GRAS/.

Francis, G. A., & O'Beirne, D. (1998). Effects of the indigenous microflora of minimally processed lettuce on the survival and growth of *Listeria innocua*. *International Journal of Food Science and Technology*, *33*, 477−488.

Griffin, P. M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Journal of the American Medical Association*, *169*, 2217−2224.

Gross, E., & Morell, J. L. (1971). The structure of Nisin. *Journal of the American Chemical Society*, *93*, 4634−4635.

Gyawali, R., Ibrahim, S. A., Hasfa, S. H. A., Smqadri, S. Q., & Haik, Y. (2011). Antimicrobial activity of copper alone and in combination with lactic acid against *Escherichia coli* O157:H7 in laboratory medium and on the surface of lettuce and tomatoes. *Journal of Pathogens*, *2011*. Article ID 650968, 9 pages. http://dx.doi.org/10.4061/2011/650968.

Hanes, D. E., Worobo, R. W., Orlandi, P. A., Burr, D. H., Miliotis, M. D., Robl, M. G., et al. (2002). Inactivation of *Cryptosporidium parvum* oocysts in fresh apple cider by UV irradiation. *Applied and Environmental Microbiology*, *68*, 4168−4172.

Hu, A. C., & Shelef, L. (1996). Influence of fat content and preservatives on the behavior of *Listeria monocytogenes* in beaker sausage. *Journal of Food Safety*, *16*(3), 176−181.

Huang, Y., & Chen, H. (2011). Effect of organic acids, hydrogen peroxide and mild heat on inactivation of *Escherichia coli* O157:H7 on baby spinach. *Food Control*, *22*, 1178−1183.

Hurst, A., & Hoover, D. J. (1993). Nisin. In P. M. Davidson, & A. L. Branen (Eds.), *Antimicrobials in foods* (pp. 369−394). New York: Marcel Dekker, Inc.

Ibrahim, S. A. H., Yang, B., & Seo, C. W. (2008). Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. *Food Chemistry*, *109*, 137−143.

Imlay, J. A., Chin, S. M., & Linn, S. (1988). Toxic DNA-damage by hydrogen peroxide through the fenton reaction in vivo and in vitro. *Science*, *240*, 640−642.

Imlay, J. A., & Linn, S. (1986). Bimodial pathern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. *Journal of Bacteriology*, *166*, 519−527.

Juliano, C., Mattana, A., & Usai, M. (2000). Composition and in vitro antimicrobial activity of the essential oil of Thymus herba-barona Loisel growing wild in Sardinia. *Journal of Essential Oil Research*, *12*, 516−522.

Jung, G. (1991a). Lantibiotics-ribosomally synthesized biologically active polypeptides containing sulfide bridges and Â-didehydroamino acids. *Angewandte Chemie, International Edition in English*, *30*, 1051−1068.

Jung, G. (1991b). Lantibiotics: a survey. In G. Jung, & H. G. Sahl (Eds.), *Nisin and novel lantibiotics* (pp. 1−34). Leiden, BV: ESCOM Science Publishers.

Kokkini, S., Karousou, R., Dardioti, A., Krigas, N., & Lanaras, T. (1997). Autumn essential oils of Greek oregano. *Phytochemistry*, *44*(5), 883−886.

Koutchma, T., Keller, S., Chirtel, S., & Parisi, B. (2004). Ultraviolet disinfection of juice products in laminar and turbulent flow reactors. *Innovative Food Science & Emerging Technologies*, *5*, 179−189.

Koutchma, T., & Parisi, B. (2004). Biodosimetry of *Escherichia coli* UV inactivation in model juices with regard to dose distribution in annular UV reactors. *Journal of Food Science*, *69*, 14.

Lawrence, B. M. (1984). The botanical and chemical aspects of oregano. *Perfumer & Flavorist*, *9*, 41−51.

Lens-Lisbonne, C., Cremieux, A., Maillard, C., & Balansard, G. (1987). Methodes d'evaluation de l'activité antibacterienne des huiles essentielles: application aux essences de thymet de cannelle. *Journal de Pharmacie de Belgique*, *42*(5), 297−302.

Marino, M., Bersani, C., & Comi, G. (2001). Impedance measurements to study the antimicrobial activity of essential oils from Lamiacea and Compositae. *International Journal of Food Microbiology*, *67*, 187−195.

Marino, M., Bersani, C., & Comi, G. (1999). Antimicrobial activity of the essential oils of Thymus vulgaris L. measured using a bioimpedometric method. *Journal of Food Protection*, *62*(9), 1017−1023.

Mass, M. R., Glass, K. A., & Doyle, M. P. (1989). Sodium lactate delays toxin production by *Clostridium botulinum* in cook-in-bag turkey products. *Applied and Environmental Microbiology*, *55*, 2226−2229.

Mazzotta, A. S. (2001). Thermal inactivation of stationary-phase and acid adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. *Journal of Food Protection*, *64*, 315−320.

McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*, *12*, 147−179.

McGimpsey, J. A., Douglas, M. H., Van Klink, J. L., Beauregard, D. A., & Perry, N. B. (1994). Seasonal variation in essential oil yield and composition from naturalized Thymus vulgaris L. in New Zealand. *Flavour and Fragrance Journal*, *9*, 347−352.

McKenna, S. M., & Davies, K. J. A. (1988). The inhibition of bacterial growth by hypochlorous acid. *Journal of Biochemistry*, *254*, 685−692.

Mukhopadhyay, S., Ukuku, D. O., Fan, X., & Juneja, V. K. (2013). Efficacy of integrated treatment of UV light and low dose gamma irradiation on inactivation of *Escherichia coli* O157:H7 and *Salmonella* enteric on grape tomatoes. *Journal of Food Science*, *78*, M1049−M1056.

Okereke, A., & Montville, T. J. (1992). Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. *Applied and Environmental Microbiology*, *58*, 2463−2467.

Olanya, O. M., Ukuku, D., Annous, B., Niemira, B., & Sommers, C. (2013). Efficacy of Pseudomonas fluorescence for biocontrol of *Escherichia coli* O157:H7 on spinach. *Journal of Food, Agriculture & Environment*, *2*, 86−91.

Padhye, N. V., & Doyle, M. P. (1992). *Escherichia coli* O157:H7: epidemiology, pathogenesis and methods for detection in food. *Journal of Food Protection*, *55*, 555−565.

Papadopoulos, L., Miller, R., Acuff, G., Lucia, L., Vanderzant, C., & Cross, H. (1991a). Consumer and trained sensory comparisons of cooked beef top rounds treated with sodium lactate. *Journal of Food Science*, *56*, 1141−1146.

Papadopoulos, L., Miller, R., Acuff, G., Lucia, L., Vanderzant, C., & Cross, H. (1991b). Effect of sodium lactate on microbial and chemical composition of cooked beef during storage. *Journal of Food Science*, *56*, 341−347.

Perumalla, A. V. S., Hettiarachchy, N. S., Over, K. F., Ricke, S. C., Gbur, E. E., Zhang, J., et al. (2012). Effect of potassium lactate and sodium diacetate combination to inhibit *Listeria monocytogenes* in low and high fat chicken and Turkey Hotdog model systems. *The Open Food Science Journal*, *6*, 16−23.

Pintore, G., Usai, M., Bradesi, P., Juliano, C., Boatto, G., Tomi, F., Chessa, M., Cerri, R., & Casanova, J. (2002). Chemical composition and antimicrobial activity of Rosmarinus officinalis L. oils from Sardinia and Corsica. *Flavour and Fragrance Journal*, *17*, 15−19.

Prudent, D., Perineau, F., Bessiere, J. M., Michel, G. M., & Baccou, J. C. (1995). Analysis of the essential oil of wild oregano from Martinique (Coleus aromaticus Benth.)—evaluation of its bacterioatatic and fungistatic properties. *Journal of Essential Oil Research*, *7*, 165−173.

Quintero-Ramos, A., Churey, J. J., Hartman, P., Barnard, J., & Worobo, R. W. (2004). Modeling of *Escherichia coli* inactivation of UV irradiation at different pH values in apple cider. *Journal of Food Protection*, *67*, 1153−1156.

Rajkowski, K. T., & Sommers, C. H. (2012). Effect of anolyte on background microflora, *Salmonella*, and *Listeria monocytogenes* on catfish fillets. *Journal of Food Protection*, *75*, 765−770.

Ray, B. (1992). Nisin of *Lactococcus lactis* ssp. lactis as a food biopreservatives. In B. Ray, & M. Daechel (Eds.), *Food biopreservatives of microbial origin* (pp. 209−264). Boca Raton, FL: CRC Press, Inc.

Ray, B., & Buhnia, A. (2008). Regulatory agencies monitoring microbiological safety of foods in the United States. In *Fund food Microbio* (4th ed., pp. 467−469). CRS Press.

Rhur, E., & Sahl, H. G. (1985). Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrobial Agents and Chemotherapy*, *27*, 841−848.

Robinson, G., Thorn, R., & Reynolds, D. (2013). The effect of long-term storage on the physiochemical and bactericidal properties of electrochemically activated solutions. *International Journal of Molecular Sciences*, *14*, 457−469.

Russo, M., Galletti, G. C., Bocchini, P., & Carnacini, A. (1998). Essential oil chemical composition of wild populations of Italian oregano spice (Origanum vulgare ssp. hirtum (Link) Ietswaart): a preliminary evaluation of their use in chemotaxonomy by cluster analysis: 1. Inflorescences. *Journal of Agricultural and Food Chemistry*, *46*, 3741−3746.

Ryser, E. T., & Marth, E. H. (1988). Survival of *Listeria monocytogenes* in cold-pack cheese food during refrigerated storage. *Journal of Food Protection*, *51*, 615−621.

Salmon, R. L., Farrell, I. D., Hutchison, J. G. P., Coleman, D. J., Gross, R. J., Fry, N. K., et al. (1989). A christening party outbreak of haemorrhagic colitis and haemolytic uraemic syndrome associated with *Escherichia coli* O157:H7. *Epidemiology and Infection*, *103*, 249−254.

Salmond, C. V., Kroll, R. G., & Booth, I. R. (1984). The effect of food preservatives on pH homeostasis in *Escherichia coli*. *Journal of General Microbiology*, *130*, 2845−2850.

Santiesteban-López, A., Palou, E., & López-Malo, A. (2006). Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected aw and pH. *Journal of Applied Microbiology*, 486−497. http://dx.doi.org/10.1111/j.1365-2672.2006.03092.x.

Sapers, G. M., Miller, R. L., & Mattrazo, A. M. (1999). Effectiveness of sanitizing agents in inactivating *Escherichia coli* in golden delicious apples. *Journal of Food Science*, *64*, 734−737.

Sapers, G. M., & Simmons, G. (1998). Hydrogen peroxide disinfection of minimally processed fruits and vegetables. *Food Technology*, *52*(2), 48−52.

Sapers, G., Walker, P., Sites, J., Annous, B., & Eblen, D. (2003). Vapor-phase decontamination of apples inoculated with *Escherichia coli*. *Journal of Food Science*, *68*, 1003−1007.

Shama, G. (1999). Ultraviolet light. In R. K. Robinson, C. A. Batt, & P. D. Patel (Eds.), *Encyclopedia of food microbiology* (pp. 2208−2214). San Diego, CA: Academic Press.

Shelef, L. A. (1994). Antimicrobial effects of lactates: a review. *Journal of Food Protection*, *57*, 445−450.

Shelef, L. A., & Seiter, J. A. (1993). Indirect antimicrobials. In P. M. Davidson, & A. L. Branen (Eds.), *Antimicrobials in foods* (2nd ed.). New York, NY: Marcel Dekker, Inc.

Shelef, L. A., & Yang, Q. (1991). Growth suppression of *Listeria monocytogenes* by lactates in broth, chicken, and beef. *Journal of Food Protection*, *54*, 283−287.

Sheu, C. W., & Freese, E. (1972). Effects of fatty acids on growth and envelope proteins of *Bacillus subtilis*. *Journal of Bacteriology*, *111*, 516−524.

Sheu, C. W., Salomon, D., Simmons, J. L., Sreeevalsan, T., & Freese, T. (1975). Inhibitory effect of lipophilic acids and related compounds on bacteria and mammalian cells. *Antimicrobial Agents and Chemotherapy*, *7*, 349−363.

Shiba, T., Wakamiya, T., Fukase, K., Ueki, Y., Teshima, T., & Nishikawa, M. (1991). Structure of the lanthionine peptides nisin, ancovenin and lanthiopeptin. In G. Jung, & H.-G. Sahl (Eds.), *Nisin and novel lantibiotics* (pp. 113−122). Leiden, BV: ESCOM Science Publisher.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., & Kokkini, S. (1996). Antimicrobial and cytotoxic activities of Origanum essential oils. *Journal of Agricultural and Food Chemistry*, *44*, 1202−1205.

Sizer, C. E., & Balasubramaniam, V. M. (1999). New intervention processes for minimally processed juices. *Food Technology*, *53*(10), 64−67.

Sofos, J. N. (1989). Mechanism of antimicrobial activity. In *Sorbate food preservatives* (pp. 11−126). Boca Raton, FL: CRC Press.

Stevens, K. A., Klapes, N. A., Sheldon, B. W., & Klaenhammer, T. R. (1992a). Antimicrobial action of nisin against *Salmonella typhimurium* lipopolysaccharide mutants. *Applied and Environmental Microbiology*, *58*, 1786−1788.

Stevens, K. A., Klapes, N. A., Sheldon, B. W., & Klaenhammer, T. R. (1992b). Effect of treatment conditions on nisin inactivation of gram-negative bacteria. *Journal of Food Protection*, *55*, 763−766.

Stevens, K. A., Sheldon, B. W., Klapes, N. A., & Klaenhammer, T. R. (1991). Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. *Applied and Environmental Microbiology*, *57*, 3613−3615.

Sung, W. S., & Lee, D. G. (2008). Mechanism of decreased susceptibility for gram-negative bacteria and synergistic effect with ampicillin of indole-3-carbinol. *Biological & Pharmaceutical Bulletin*, *31*(9), 1798−1801.

Ukuku, D.O. (1995). *Bioluminescent ATP assay for susceptibility of Listeria monocytogenes to antimicrobials* (Doctoral theses). Wayne State University. p. 204.

Ukuku, D. O. (2004). Effect of hydrogen peroxide treatment on microbial quality and appearance of whole and fresh-cut melons contaminated with *Salmonella* spp. *International Journal of Food Microbiology*, *95*, 137−146.

Ukuku, et al. (2009). *Foodborne Pathogens and Disease*, *6*(4).

Ukuku, D. O., Bari, L., & Kawamoto, S. (2013). Hydrogen peroxide. In V. M. Gomez-Lopez (Ed.), *Decontamination of fresh and minimally processed produce* (pp. 197−214). USA: Wiley-Blackwell.

Ukuku, D. O., Bari, M. L., Kawamoto, S., & Isshiki, K. (2005). Use of hydrogen peroxide in combination with nisin, sodium lactate and citric acid for reducing bacterial pathogens from whole melon surfaces to fresh-cut pieces. *International Journal of Food Microbiology*, *104*, 225−233.

Ukuku, D. O., & Fett, W. F. (2002). Effectiveness of chlorine and Nisin-EDTA treatments of whole melons and fresh-cut pieces for reducing native microflora and extending shelf-life. *Journal of Food Safety*, *22*, 231−253.

Ukuku, D. O., & Fett, W. F. (2004). Effect of Nisin in combination with EDTA, sodium lactate, potassium sorbate for reducing Salmonella on whole and fresh-cut cantaloupe. *Journal of Food Protection*, *67*, 2143−2150.

Ukuku, D. O., & Geveke, D. J. (2010). A combined treatment of UV-light and radio frequency electric field for the inactivation of *Escherichia coli* K-12 in apple juice. *International Journal of Food Microbiology*, *138*, 50−55.

Ukuku, D. O., Geveke, D. J., Zhang, H. Q., & Cooke, P. H. (2008). Membrane damage and viability loss of *E. coli* K-12 in apple juice treated with radio frequency electric fields treatment. *Journal of Food Protection*, *71*, 684−690.

Ukuku, D. O., Mukhopadhyay, S., & Olanya, M. (2013). Effect of organic acid treatments on microbial safety and overall acceptability of fresh-cut cantaloupe cubes. *Journal of Food, Agriculture & Environment*, *11*(3&4), 340−345.

Ukuku, D. O., Olanya, M., Geveke, D. J., & Sommers, C. (2012). Effect of native microflora, waiting period and storage temperature on the population of *Listeria monocytogenes* serovars transferred from cantaloupe rind surfaces to fresh cut pieces during preparation. *Journal of Food Protection*, *75*(11), 1912−1919.

Ukuku, D. O., Pilizota, V., & Sapers, G. M. (2004). Effect of hot water and hydrogen peroxide treatments on survival of *Salmonella* and microbial quality whole and fresh-cut cantaloupes. *Journal of Food Protection*, *67*, 432−437.

Ukuku, D. O., & Sapers, G. M. (2001). Effect of sanitizer treatments on *Salmonella* stanley attached to the surface of cantaloupe and cell transfer to fresh-cut tissues during cutting practices. *Journal of Food Protection*, *64*, 1286−1291.

Ukuku, D. O., & Sapers, G. M. (2007). Effect of time before storage and storage temperature on survival of *Salmonella* inoculated on fresh-cut melons. *Food Microbiology*, *24*, 288−295.

Ukuku, D. O., & Shelef, L. A. (1994). *ATP depletion and susceptibility of* Listeria monocytogenes *to nisin, ampicillin, and sodium propionates*. Program Abstr. Abstr. 59-E 1. Atlanta, GA: I.F.T. Annual Meeting.

Ukuku, D. O., & Shelef, L. A. (1996). Bioluminescence measurements of the antilisterial activity of nisin: comparison with ampicillin and streptomycin. *Journal of Bioluminescence and Chemiluminescence*, *11*, 169−173.

Ukuku, D. O., & Shelef, L. A. (1997). Sensitivity of six strains of *Listeria monocytogenes* to nisin in broth at pH 7, 6, and 5. *Journal of Food Protection*, *60*, 867−869.

Ukuku, D. O., Zhang, H. Q., & Huang, L. (2009). Growth parameters of *Escherichia coli, Salmonella* enteritidis and *Listeria monocytogenes*, and Aerobic Mesophilic Bacteria of Apple cider amended with nisin-EDTA. *Foodborne Pathogens and Disease*, *6*, 487−494.

Virto, P., Manas, P., Alvarez, I., Condon, S., & Raso, J. (2005). Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Applied and Environmental Microbiology*, *71*, 5022−5028.

Wei, C.-I., Cook, D. L., & Kirk, J. R. (1985). Use of chlorine compounds in the food industry. *Food Techonology*, *1*, 107−115.

Woo, I.-S., Rhee, I.-K., & Park, H.-D. (2000). Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. *Applied and Environmental Microbiology*, *66*, 2243−2247.

Woolford, E. R., & Anderson, A. A. (1945). Propionates control microbial growth in fruits and vegetables. *Food Industry*, *17*, 622−628.

Wright, J. R., Sumner, S. S., Hackney, C. R., Pierson, M. D., & Zoecklein, B. W. (2000). Efficacy of ultraviolet light for reducing *Escherichia coli* O157:H7 in unpasteurized apple cider. *Journal of Food Protection*, *63*, 563−567.

Yildiz, F. (1994). Chapter 2 − Initial preparation, handling, and distribution of minimally processed refrigerated fruits and vegetables. In R. C. Wiley (Ed.), *Minimally processed refrigerated fruits and vegetables*. New York: Chapman and Hall.

Physical and chemical methods for food preservation using natural antimicrobials

10

C. Barry-Ryan
Dublin Institute of Technology, Dublin, Ireland

10.1 Introduction

The shelf life of perishable food products is determined by the onset of spoilage during their preparation, distribution and storage, and many food products are now sold far away from their production sites. Refrigeration and advances in the cold distribution chain have improved shelf life and exportability but cannot ensure the quality and safety of all perishable foods. Therefore, there is a requirement for the use of antimicrobial agents and preservatives. However, there is a growing consumer awareness and concern regarding synthetic chemical additives; examples of such significant antimicrobial agents that have aroused concern include nitrites (Xi, Sullivan, Jackson, Zhou, & Sebranek, 2011), sulphites (Hardisson, Rubio, Frıas, Rodrıguez, & Reguera, 2002) and chlorine (Ahmed, Martin-Diana, Rico, & Barry-Ryan, 2013).

Research and commercial applications have shown that natural components could replace traditional sanitising agents. This chapter reviews the range of these natural antimicrobials, their applications and the efficacies of these applications. The direct application of natural antimicrobials to food includes both chemical and biological agents and physical intervention methods, such as high-pressure processing, essential oils and bacteriocins.

10.2 Physical application of natural antimicrobials

10.2.1 Water and washing

Once slaughtered or harvested, some initial pre-processing of food commodities must occur to reduce surface contaminants such as dirt and debris and possibly reduce epithelial microflora. Such steps include initial rinsing and trimming but may include peeling for horticultural commodities, de-scaling for fish, and hair removal or evisceration for animal products. Washing in water is an essential step in preparing produce (whole or processed) products for consumption; it reduces cross-contamination by removing dust and soil particles that harbour many micro-organisms. Such cleaning steps are used for a range of raw materials for food production and may include soaking (e.g. potatoes), spraying (e.g. animal carcasses), flotation washing (e.g. apples); dry cleaning

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procedures include separation by air (e.g. cereal grains), magnetism (for metal removal) or physical methods (e.g. centrifugal clarification of milk). However, wash water harbours residual dirt and microflora and therefore need antimicrobial agents (e.g. chlorine) to be added or water treatment (e.g. filtration) to occur before reuse or dumping.

Spraying is a very effective method for washing because it can be targeted (e.g. to soft rot spots on peaches), the pressure can be controlled (high pressure for biofilm removal) and the temperature is adjusted according to the product. Spraying with hot water or steam combines partial heat decontamination with mechanical removal of bacteria. Such treatment is limited by the heat sensitivity of the product's surface, but the water temperature must be high enough to kill the bacteria (Pipek, Sikulova, Jelenıkova, & Izumimoto, 2005). Steaming has potential for industrial application; exposure times should be kept short. The application of hot steam with added organic acids such as lactic acid by spraying is an effective surface decontaminant of animal carcasses (Pipek, Houska et al., 2005).

Electrolysed water is generated via the electrolysis of diluted sodium chloride, producing an electrolysed basic and acidic solution at the cathode and anode sites, respectively (Goodburn & Wallace, 2012). Gómez-López et al. (2007) and Rico et al. (2008) found that neutral electrolysed water was comparable with standard chlorine for reducing specific microbial loads such as psychotrophs on processed cabbage (equivalent to 50 ppm free Cl^-) or mesophiles on lettuce (equivalent to 60 ppm free Cl^-). Issa-Zacharia, Kamitani, Miwa, Muhimbula, and Iwasaki (2011) reported similar activity for the slightly acidified electrolysed water (lower free Cl^- of 20 ppm) for ready-to-eat vegetables and sprouts compared with a traditional chlorine wash (120 ppm), with a reduction in *Escherichia coli*, *Salmonella* spp. and total aerobic mesophilic bacterial counts. Acidic electrolysed water with a low pH value was associated with a high oxidation and reduction capacity (equivalent to 20 ppm free Cl^-) and was more effective than chlorine in combating *E. coli* (Keskinen, Burke, & Annous, 2009). Various types of washers are available to the food industry and are designed and adapted to the characteristics and special requirements of specific commodities (Sapers, 2009). Washers may be either immersion or non-immersion. Immersion washers work by submerging the product in agitated water, which can be created by using water, air, sound or mechanical devices such as stirrers, baffles and paddles that can be wood, plastic or metal. There are many types of mechanical devices, including dump tank or flume systems, hydro-air or bubble washers, paddle washers and drum, basket or barrel washers. The various types of immersion systems have inherent advantages and disadvantages, and some systems are applicable to only certain classes of food products. Non-immersion washers work by spraying or rinsing the product with a washing solution. There are three types of non-immersion washers: roller brush, high-pressure and drum/basket/barrel non-immersion washers (Pao, Kelsey, & Long, 2009).

10.2.2 Packaging

The principles of packaging are to offer protection to products from physical, physiological and pathological deterioration throughout the distribution chain; to immobilise the product; and to promote the product during marketing.

Modified atmosphere packaging (MAP) removes the air surrounding the food product and replaces it with an appropriate atmosphere before sealing it in a suitable film material to inhibit the growth of spoilage and pathogenic micro-organisms. Gas combinations vary depending on the commodity packaged; fruits and vegetables and products are typical packaged with low oxygen (5%) and elevated carbon dioxide (5−10%). The majority of MAP for fresh meat requires a highly oxygenic environment (e.g. 80%) to retain the fresh red colour and give a sufficient shelf life within a controlled distribution system. Vacuum packaging removes most of the air before the product is enclosed in barrier materials, whereas gas replacement removes the air by vacuum or flushing and replaces it with another gas mixture before sealing the packaging. The headspace environment and the product may change during storage in MAP, but there is no additional manipulation of the internal environment, whereas controlled atmosphere packaging uses continuous monitoring and control of the environment to maintain a stable gas atmosphere and other conditions such as temperature and humidity within the package.

Active packaging (intelligent packaging or smart films) interacts with the product or the headspace between the package and the food system to control the atmosphere and or microbial growth (Brody, Strupinsky, & Kline, 2001). For example, oxygen scavengers (e.g. iron based) or carbon dioxide generators (e.g. carbonate based) in sachets are used to create low-oxygen atmospheres in the package to control microbial growth or slow the oxidation of lipids. Active antimicrobial food packaging can also inhibit or retard the growth of micro-organisms. It can disperse the active agent within the packaging film, coating the packaging material or be manipulated to have film-forming properties itself (e.g. chitosan, as described by Kong, Chen, Xing, and Park (2010)). Consideration has to be given to the amounts of active compound migrating from the packaging material to make this technique compatible with current legislation. To be incorporated into the plastic films, these active compounds may need to be thermostable.

10.2.3 Temperature

10.2.3.1 Low temperature

Pre-cooling (or fast cooling) is the process by which field heat from horticultural crops is rapidly removed. It is essential that field heat be removed soon after the harvest of several crops to control quality and microbial proliferation. Various methods are used, including (1) forced-air cooling for packed commodities; (2) hydro-cooling for roots and stems (e.g. cold water spray); (3) vacuum cooling, which is very effective for products with a large surface area (e.g. lettuce) and (4) evaporative cooling using air or simply packaging in ice (transport and salt). Chilling meat is essential for maintaining quality, including tenderness, microbial safety and yield. Large red meat carcasses are typically chilled by air while suspended on an overhead shackle line (held for 6−24 h), whereas smaller broiler/turkey carcasses can be chilled either by air or water. In the case of water chilling, carcasses can be suspended from a shackle line or dropped into a long screw/paddle-type cold water bath and moved through to the end (speed can

be adjusted to control chilling duration). Counter-flow patterns are used (clean cold water flow from the exit side) to improve the efficiency and hygiene of the meat (Barbut, 2014).

Freezing involves reducing the temperature of the product by removing latent heat, which requires energy and time to crystalise water. The advantage of freezing is it prolongs the shelf life of the commodity by controlling microbial and chemical changes. The faster the freezing rate, the smaller the intracellular ice crystals and less drip loss during thawing and cooking. The size of the product, its thermal properties (e.g. specific heat and thermal conductivity), environmental temperature, method of application and packaging material all affect the rate of freezing. Cryogenic freezing is a more rapid method of freezing and requires only a cryogen tank and suitable spray equipment. The commercial application of cryogenic freezing may be limited because the shape of the product maybe distorted and the cost of cryogenic liquid is relatively high.

Superchilling is the reduction of the temperature of the product to just 1−2 °C below the product's initial freezing point. Mechanical, cryogenic or impingement freezers may be used. Then the ice distribution and temperature of the product equilibrates and is maintained during storage and distribution. Superchilling maintains food freshness, retains high quality, suppresses the growth of harmful microbes, thereby increasing yield and reducing energy and labour costs, but some chemical and physical changes may occur during storage. Superchilling may also lead to reduced transport costs, easier handling during processing and reduced environmental impact. Superchilling has been successfully used for seafood; in 2011 Claussen reported that the shelf life of salmon was increased by 50% using superchilling compared with the chilled product.

Whatever the antimicrobial and preservation methods chosen, the cold chain, which starts with product processing and production and ends at cold stores in retail cabinets, is essential in maintaining the quality and safety of foods. A cold chain management system consists of processes, facilities and information that keep products at a low temperature during all stages of production (Aung & Chang, 2014). Maintaining an optimum temperature during transportation is a difficult task and is hard to regulate throughout. Sea, air and land transportation systems are expected to maintain the temperature of the food within correct limits to ensure optimum safety and prolonged shelf life (James & James, 2010).

Monitoring the cold chain process is also an issue, especially during storage and transportation. There is an increasing demand for traceability in the cold chain; statutory requirements are growing stricter and there are increased demands to develop standardised traceability systems. Throughout the cold chain, more details, such as humidity or information about tampering, is required. Sensors such as radiofrequency identification sensor tags are replacing the traditional manual recording and digital loggers of temperature, pressure, humidity, inclination and acceleration (Aung & Chang, 2014).

10.2.3.2 Heat treatment

Heat treatment factors (time/temperature regime) can vary to accomplish almost any degree of microbial inactivation, ranging from limited reduction of microbial load to complete sterilisation. In terms of microbial inactivation, these heat treatments are

characterised using values such as the D value (decimal reduction time, which is the time of the heat treatment in minutes and gives an expected microbial inactivation of 90%); the Z value (temperature change required for a 10-fold change in D value); and F value (the reference time in minutes at a reference temperature of 121 °C for the desired sterilisation effect). Retorting or canning is generally conducted at 121 °C for 25−40 min or at 115 °C for 30−40 min to produce a sterile food product and ensure destruction of *Clostridium botulinum* spores. Pasteurisation occurs at lower temperatures (e.g. 60−85 °C for milk) and is associated with the destruction of vegetative cells of micro-organisms (Rajkovic, Smigic, & Devlieghere, 2010).

These thermal methods are widely used for the preservation of foods but can lead to undesirable changes such as loss of vitamins and colour and the formation of thermal reaction components that can affect appearance, flavour and texture. To reduce these undesirable changes in quality, the use of high temperature for a short time has been successful. Micro-organisms are inactivated at the temperature of the heat treatment, and the undesirable quality changes depending on the length of the heat treatment. Therefore, high temperatures rapidly inactivate micro-organisms, and short times give fewer undesired changes in quality (Ohlsson, 2002). Heat-shock is a high temperature, short time method applied to horticultural products that usually involves a washing step at a temperature range of 45−70 °C for less than 5 min. The use of heat-shock is reported to enhance the quality and bactericidal effect of sanitisers (Martin-Diana, Rico, Frías et al., 2006).

Heat treatment can also be used to decontaminate and disinfest produce; there are three popular methods used: vapour heat treatment, forced hot-air treatment and hot water immersion treatment. Some may be very brief and use brushes. The timing of the application of heat treatment (before or after processing) is very important for maximizing its beneficial effects (Barry-Ryan, 2012).

Blanching is well established as a decontaminant treatment in the fruit and vegetable industries. It involves heating the product at high temperatures, generally in water at 85−100 °C or with steam−less frequently with microwaves, radiofrequency or infrared radiation−for short exposure times. However, blanching can have deleterious effects, including the loss of nutrients, texture and colour, and it requires large amounts of power and generates effluents (Song, An, & Kim, 2003).

Steamer-jet injection uses short steam processing (10 s) and was successfully used as an alternative to chlorine (100 mg/kg) in control of mesophilic micro-organisms on fresh-cut lettuce (Martín-Diana, Rico, Mulcahy et al., 2006).

A vacuum-steam-vacuum system is a process that kills bacteria without thermal degradation. It normally uses a short total process time of 0.5−1.2 s, depending on the number of cycles and the process time per cycle. The vacuum-steam-vacuum system was successfully applied to cantaloupes, grapefruits, papayas, mangoes, avocados, kiwis, carrots, cucumbers, peaches and beets, reducing inoculated *Listeria innocua* and total aerobic plate counts. However, applying this process to bananas, cauliflower, broccoli and peppers resulted in thermal or mechanical damage. The surface of the produce is heated rapidly using a steam jet inside a vacuum. The initial vacuum removes any layers of gas that can impair the heat transfer, and the final vacuum cools the produce by evaporating the water that may have condensed from the steam. Cycling

between vacuum and steam effectively removes this redeposited water layer as soon as it forms and improves surface treatment (Kozempel, Radewonuk, Scullen, & Goldberg, 2002).

Drying or dehydration includes removal of water from the solids of the food commodity to a level at which microbial spoilage is minimised, increasing stability during long-term storage. Water can be removed by freeze drying, spray drying or cabinet drying.

Radiofrequency heating consists of dielectrically heating the product through radio waves. This current is used to create electromagnetic radiation, which produces heat within the product when placed between electrodes with alternating polarity. The oscillation of ions and water in a food product inside the electric field generates heat by friction and thus inactivates micro-organisms present (Marra, Zhang, & Lyng, 2009).

10.2.4 Other physical antimicrobial methods

10.2.4.1 Light

Ultraviolet (UV) light can induce damage to DNA and indirectly induce resistance mechanisms in different pathogens, making it an effective antimicrobial agent. The advantage of UV light is it is relatively inexpensive and easy-to-use equipment is needed, but it may have some deleterious effects on the product (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000).

Pulsed light (PL) is a non-thermal technology that uses pulses of short, intense broad-spectrum light to inactivate pathogens. The application of intense PL is an emerging technology in food preservation. This technique also has been investigated as a decontamination agent of equipment in contact with food (Goodburn & Wallace, 2012). The pulses are a continuous spectrum (200- to 1100-nm wavelengths) rich in UV light that last a few hundred microseconds. The mechanism of microbial inactivation by PL occurs through a photochemical effect, inducing structural changes in DNA and the agglutination of cytoplasmatic content, leading to a disruption of cell membranes. Thus, significant and rapid microbial inactivation in short treatments, lack of residual compounds and great flexibility are some of the advantages of this technology for food application (Aguiló-Aguayo, Charles, Renard, Page, & Carlin, 2013). The disadvantage is the possible side effects of heat on nutritional or sensorial properties of food.

10.2.4.2 Ultrasound

Ultrasound is the use of sound waves with a frequency of 20 kHz or more; generally, ultrasound equipment uses frequencies from 20 kHz to 10 MHz. The application of ultrasound is a non-thermal technology that contributes to the increase of microbial safety and prolongs shelf life, especially in food with heat-sensitive, nutritional, sensory, and functional characteristics (O'Donnell, Tiwari, Bourke, & Cullen, 2010). A major advantage of ultrasound over other techniques in the food industry is that sound waves are generally considered safe, non-toxic and environment-friendly. Higher-power

ultrasound at lower frequencies (20−100 kHz) has the ability to cause cavitation, which could be used to inactivate micro-organisms. Cavitation consists of the formation of bubbles inside a liquid, which implode, causing cell damage. The mechanism of microbial killing is mainly due to the thinning of the cell membranes, localised heating and free radical production (Chemat, Zill-e-Huma, & Khan, 2011).

10.2.4.3 High pressure

By subjecting foods to high pressures in the range 3000−8000 bars, micro-organisms and enzymes can be inactivated without the degradation of flavour and nutrients associated with traditional thermal processing (Palou, Lopez-Malo, Barbosa-Canovas, & Welti-Chanes, 2000). A steel cylinder containing a liquid pressure-transmitting medium (usually water) and the sample, which can be protected using appropriate packaging, is used for high-pressure processing. The sample is maintained under pressure for an extended period of time, and this does not require any additional energy to maintain the chosen temperature.

There are some problems associated with high pressure use because it can affect the integrity of porous products. The air confined in the food matrix is subjected to compression and expansion during pressurisation and decompression, disrupting food tissues; this therefore makes operation of this unit unsuitable for certain products such as fresh vegetables (Palou et al., 2000).

10.2.4.4 Photosensitisation and electrostatic spraying

Photosensitisation is another non-thermal method used to decontaminate the surface of produce. It involves the accumulation of a photoactive compound in the bacteria, followed by illumination with visible light, which destroys the bacterial cells in the presence of oxygen. The main advantage of photosensitisation compared with other antibacterial tools is the absence of any bacterial resistance to this treatment, and it has minimal destructive effects on the surrounding matrix. Haematoporphyrin, sodium chlorophyllin, riboflavin and psoralen are photodynamic active plant food constituents that could be used as photosensitizers for foods. Food pathogens *Listeria monocytogenes* and *Bacillius cereus* were effectively inactivated by Na-chlorophyllin-based photosensitisation *in vitro* and on the biofilms and spores on the surface of polyolefin packaging material and was more effective than washing with water or 200-ppm Na-hypochlorite. The potential of these photodynamic active components in food preservation need more research to evaluate its future in food preservation (Luksiene & Paskeviciute, 2011).

Electrostatic spraying is an emerging technology for decontamination whereby the surface of food can be coated with a fine layer of an antimicrobial substance. An intense electric field is applied to the surface of the antimicrobial liquid; this induces an electrostatic force sufficient to overcome the surface tension and disrupts the liquid so that it becomes a spray of charged particles. This disruption of the droplet surface becomes a cloud of charged droplets, which are attracted to the surface. This technology can utilise organic acids, and its antimicrobial activity has been shown to be comparable to chlorine (Kashif, Khan, Schutyser, Schroën, & Boom, 2012). Benefits of this technology are that organic acids are less corrosive and cause less damage to

processing equipment compared with electrolysed or conventional chlorine spraying systems. In addition, organic acids have occupational health benefits for processing staff in that they are easier to use and are more stable (Goodburn & Wallace, 2012).

10.3 Chemical application of natural antimicrobials

10.3.1 Herbs, spices and their essential oils

Herbs and spices were originally added to change or improve the taste of food, but they can also enhance shelf life because of their antimicrobial nature. Spices (woody stemmed plants), which have strong antimicrobial activity, include allspice, cinnamon, clove, mustard and vanillin. Among herbs (green stemmed plants), basil, oregano, rosemary, sage and thyme have the most potent antimicrobial action. Natural antimicrobials have been identified in the essential oils of herbs and spices (Goodburn & Wallace, 2012).

Essential oils consist of concentrated aroma compounds and are volatile or ethereal oils that are usually extracted from plant materials such as leaves, bark or fruit (Oussalah, Caillet, Saucier, & Lacroix, 2006). These essential oils occur in edible, medicinal and herbal plants, which reduce concerns for their safe use in food products. Essential oils and their constituents have been widely used as flavouring agents for a long time, and their wide spectra of antimicrobial action is well established (Burt, 2004). Plant essential oils serve as alternative sanitisers with generally recognised as safe status (Gutierrez, Barry-Ryan, & Bourke, 2008). They present a broad spectrum of antimicrobial activity useful for reducing the risk of contamination associated with food-borne pathogens (Gutierrez, Rodriguez, Barry-Ryan, & Bourke, 2008). The high efficacy of essential oils against spoilage micro-organisms and specific pathogens has been reported in various studies (Gutierrez, Rodriguez et al., 2008; Hammer, Carson, & Riley, 1999). Gram-negative bacteria are slightly less susceptible to antimicrobials than Gram-positive organisms because of lipopolysaccharides present within the outer membrane (Burt, 2004).

The levels of inactivation differs between oils; inactivation is highly concentration dependent, has differing mechanisms of action and is dependent on any possible adaptation by the organism. Clove, oregano, rosemary, thyme, sage and vanillin have been found to be the most consistently effective oils against micro-organisms. Their composition, structure and functional groups play an important role in determining their antimicrobial activity. Compounds with phenolic groups usually are most effective (Dorman & Deans, 2000). For natural oils to be an innovative tool for microbiological reduction, further work is needed to understand both which oils can be effective against the target pathogen and their organoleptic acceptability, in terms of tainting and toxicity. The application of these lipophilic antimicrobials, washing times and temperature need to be reviewed for industrial application and acceptability.

10.3.2 Smoke

Smoke has been successfully applied in food preservation for a long time, often in conjunction with other hurdles such as cooking and drying. It is used to impart flavour,

colour and aroma to foods. Natural wood smoke generated by controlled smouldering of wood in the absence of or at reduced levels of oxygen is a suspension of vapours, solid particles and liquid droplets (Holley & Patel, 2005). Because of concern regarding the presence of potentially carcinogenic benzopyrenes and because of convenience issues, liquid smoke was developed and has become popular. For the manufacture of liquid smoke, smoke from smouldering wood is passed to a condensing tower, where it is captured in water and filtered. Smoke flavours can be used in foods as an additional barrier to prevent microbial growth at levels that comply with good manufacturing practices. Food composition plays an important role in determining the overall antimicrobial activities of smoke solutions (Sunen, Fernandez-Galian, & Aristimuno, 2001). Product handling, smoking temperature and relative humidity in smokehouses can be controlled to exert additional pressure on microbial contaminants.

A more recent development in smoke technology is the commercialisation of liquid smoke preparations, which are either soya or canola oil based rather than aqueous based. The oils extract flavours from liquid smoke, facilitating the transfer of oil-soluble phenolics but leaving behind the less soluble tangy acids; they have a very mild flavour because a larger portion of the carbonyls is not extracted and therefore are suitable for foods with a delicate flavour character, such as fermented sausage and cheese. They are better antioxidants but have reduced capacity to generate colour. Liquid smoke usually contains acetic acid, which is regarded as being responsible for a large portion of its bacteriostatic properties. The antimicrobial activity of liquid smoke is also attributed to the presence of compounds such as phenols, carbonyls and organic acids (Vitt, Himelbloom, & Crapo, 2001). The concentration of polar phenolic compounds present, which have higher water solubility and therefore have greater opportunity to contact and interact with target organisms, determine the levels of kill.

10.3.3 Natural nitrate

The growing concerns about nitrite in food have prompted consumer demands for uncured, no nitrate/nitrite-added meat and poultry products (Xi et al., 2011). Replacing conventional sodium nitrate and nitrite to simulate typical curing has successfully been achieved using a natural source of nitrate, such as celery or cranberry powder, and a bacterial starter culture to reduce nitrate to nitrite during the manufacturing process. Some processors are now using pre-converted powder/juice in which nitrate has already been converted to nitrite by suppliers (Sebranek & Bacus, 2007). Regulations require that all of these products be labelled as ‘uncured’ because nitrite or nitrate is not added directly to the meat mixture (Sebranek & Bacus, 2007). However, a more technically correct term could be ‘naturally cured’. The impact of these powders on sensory quality attributes of processed meats is an important consideration that should be taken into account before their antimicrobial properties can be effectively utilised (Xi et al., 2011).

10.3.4 Edible films and coatings

Edible films and coatings are transparent layers that coat the food and can be prepared either individually or from a combination of different components. They may be

polysaccharide based (cellulose, chitosan, starch, alginate, pectin and dextrin) or protein-based (wheat gluten, collagen, soy, corn zein, casein and whey protein) and contain lipid-based components (waxes, acylglycerols and fatty acids). Coatings may be added as a thin layer on the surface of food or on different layers of food components (Karbowiak, Debeaufort, Voilley, & Trystram, 2009). Edible coatings and films act as a barrier against moisture, gases and volatile substances but can also be used as food additives, such as flavouring, antioxidants, vitamins and colours. Their anti-browning nutritional properties and antimicrobial activities have recently been demonstrated, reducing the risk of pathogen growth (Rojas-Grau, Soliva-Fortuny, & Martın-Belloso, 2009).

Chitosan, a cationic polysaccharide with a high molecular weight, is a linear polymer that comprises β-1,4-linked glucosamine with various amounts of N-acetylated β-1,4-linked glucosamine residues. It is typically obtained by the alkaline deacetylation of chitin extracted from shellfish exoskeletons or the cell walls of some micro-organisms and fungi. It has antibacterial and antifungal activity, which is directly linked to its molecular weight; chitosan with a molecular weight in the range of 5−20 kDa has greater biological activity than total chitosan (Muzzarelli & Muzzarelli, 2002). When applied as an edible coating to carrots, it demonstrated the ability to control the growth of mesophilic, psychotropic micro-organisms, in addition to yeast and moulds (Durango, Soares, & Andrade, 2006).

Whey permeate is a by-product of the production of whey protein concentrate from cheese whey. The main components of whey permeate are water, lactose, peptides and minerals, and it is a promising natural bioactive alternative for the preservation of fresh produce (Ahmed, Martin-Diana, Rico, & Barry-Ryan, 2011a,b). Whey antimicrobial peptides were reported to act against different Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Fitzgerald & Murray, 2006). Milk proteins identified as having antimicrobial properties include lactoferrin, kappacin and lysozyme, and their activity is based on their inherent amino acid composition and sequence. The size of active sequences may vary from 2 to 20 amino acid residues, and many peptides are known to reveal multi-functional properties (Korhonen, 2009).

10.3.5 Organic acids

Many organic acids have been found to be microbiostatic and can affect fungi and bacterial cells. Therefore, organic acids and their salts are added to food to control the pH, to prevent oxidation or to serve as preservatives, for example, lactic, acetic, ascorbic, benzoic, citric and propionic acids. Organic acids might be applied as sprays, washes or dipping solutions, depending on the food product to be decontaminated and the infrastructure of the processing plant. The preservative mechanism occurs by reduction of environmental pH, disruption of membrane transport/permeability, anion accumulation or a reduction in internal cellular pH. The micro-organism tries to maintain its internal pH, extruding organic acids or protons, thereby using all its energy. In addition, it has been proposed that short-chain fatty acids, such as sorbic, propionic and benzoic acids, influence the membrane either by interfering with the membrane proteins or by changing the membrane fluidity (Eklund, Poysky, & Habig, 1989). Other

modes of action include inactivating or affecting metabolic enzymes, protein synthesis systems or genetic material (Vega-Mercado et al., 1997). Surface decontamination of carcasses using lactic acid is often used because it is a natural compound produced during postmortem glycolysis in meat. In addition, lactate anions inhibit the growth of surviving microbes during storage (Pipek, Sikulova, et al., 2005).

Organic acids such as lactic, citric and acetic acids at a concentration of 1.5−2.5% have been approved as acceptable for reducing microbial pathogens on meat carcasses in the United States; in the European Union they are still not fully authorised but are under consideration (Rajkovic et al., 2010).

The antibacterial properties of calcium propionate for the treatment of honeydew melon was reported and attributed to its ability to uncouple microbial transport processes (Saftner, Baj, Abbott, & Lee, 2003). Calcium lactate was tested as a sanitiser of fresh-cut lettuce and carrots and was comparable to chlorine (Martín-Diana et al., 2005) and did not affect the quality of the product. The use of calcium-based treatments presents a further advantage; in some cases it can significantly increase the calcium content of the final product (Anino, Salvatori, & Alzamora, 2006), which might enhance the appreciation of these products since the awareness of consumers of the benefits of calcium is relatively high.

10.3.6 Ozone

Ozone (O_3) has been recognised as a strong antimicrobial agent and is used in the treatment of drinking water. It was approved by the US Food and Drug Administration in 2001 as an decontaminant for minimally processed fruits and vegetables (Jurado-Alameda, García-Román, Altmajer-Vaz, & Jiménez-Pérez, 2012). The mode of action for O_3 is either directly by reaction with molecular O_3 or indirectly as free radicals ($\bullet OH$ and $H_2O\bullet$), which are derived from O_3. O_3 must be generated on site because it decomposes quickly into water and oxygen. It does not form by-products and exhibits greater oxidation activity than chlorine (Smilanick, Crisosto, & Mlikota, 1999). As a decontaminant, O_3 is subject to less regulatory concerns because of its production of aldehydes, ketones and carboxylic acid in the presence of organic matter. The major disadvantage of O_3 application is related to the safety concerns of staff who are working with it (exposure limit is 0.1 ppm), in addition to the high cost of its generation (Rico, Martın-Diana, Barat, & Barry-Ryan, 2007).

10.4 Biological application of natural antimicrobials

10.4.1 Bacteriocins

Bacteriocins are cationic antimicrobial peptides produced by a variety of bacteria that can reduce the microbial population when applied during washing treatment (Abriouel, Benomar, Lucas, & Gálvez, 2011). Several studies indicate that the bacteriocins produced by lactic acid bacteria (LAB), or *Bacillus* species, are bio-protective in relation to fruits and vegetables (Abriouel et al., 2011). The effectiveness of bacteriocins as

food preservatives is well proven, and purified nisin and preparations of pediocin are used commercially (e.g. ALTA 2351). Although bacteriocins are inhibitory against food-borne pathogens such as *L. monocytogenes*, their synthesis and mode of action distinguish them from clinical antibiotics (Cleveland, Montville, Nes, & Chikindas, 2001).

10.4.2 Bacteriophages

A bacteriophage particle is composed of a core nucleic acid, which may be double- or single-stranded DNA or RNA, coated with a protein shell, which forms the capsid (Ackermann & Kropinsk, 2007). A wide diversity of bacterial viruses or bacteriophages exists; thousands of phages have been characterised using electron microscopy. Bacteriophages have been investigated as potential anti-pathogen agents (Parish et al., 2003; Ye, Kostrzynka, Dunfield, & Warriner, 2009, 2010). Lytic bacteriophages that attack and lyse bacterial cells have the potential to function as natural methods of controlling the micro-organism population in food (Leverentz et al., 2011; Oliveira et al., 2014). Bacteriophages are ubiquitous in the environment, and their specific targeting of food-borne pathogens could be useful for preservation without changing the microbial ecology of produce. However, the practicality of this technology in an industrial setting with regard to incubation temperatures and their likely impact on produce quality needs to be evaluated. The specificity of bacteriophages restricts their potential for use when multiple pathogens may be present as contaminants (Goodburn & Wallace, 2012).

10.4.3 Protective cultures

Microbial antagonism can be used to control other populations of micro-organisms using different rates of growth, competition for space and nutrition or creating antimicrobial substances between competitors (Cleveland, Montville, Nes, & Chikindas, 2001). *Pseudomonas* spp. have been recognised as biocontrol agents in the spoilage of fruits and vegetables, and products are commercially available (e.g. Biosave) (Mikani et al., 2008).

10.4.4 Fermentation

LAB are Gram-positive bacteria; they are anaerobic and aerobic, non-spore-forming cocci or rods that produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, such as sourdough bread, all fermented milks and most fermented vegetables. Bacteria from the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Streptococcus* are the main species involved in lactic fermentations (Caplice & Fitzgerald, 1999). LAB convert carbohydrates to lactic acid, carbon dioxide and other organic acids that contribute to a product's flavour without the need for oxygen. Some of the strains are homofermentative and others are heterofermentative and produce lactic acid, other volatile compounds and small amounts of alcohol (Gaggia, Di Gioia, Baffoni, & Biavati, 2011). The lactic acid produced by LAB is effective in inhibiting the growth of other bacteria. The large

diversity of LAB makes them very adaptable to a range of conditions and is mainly responsible for their success in food fermentations (Bourdichon et al., 2012).

10.4.5 Antimicrobial peptides from eukaryotes

Many species have developed systems of antimicrobial defense against competitors or infections: the production of antimicrobial metabolites. Antimicrobial metabolites isolated from endophytes belong to diverse structural classes, including alkaloids, peptides, steroids, terpenoids, phenols, quinones and flavonoids. These peptides act against a specific group of competing organisms, and their broad spectrum of activity serves as a more general defense mechanism. Antimicrobial peptides protect the host through different mechanisms, most commonly by permeabilising the target cell membrane, resulting in an irreversible leakage of cellular material and consequently cell death. Leuesnostatin A produced by *Acremonium* spp. in *Taxus baccata*, displayed antimicrobial activity against *Pythium ultimum* (Strobel, Torzynski, & Bollon, 1997). Cryptocandin, a peptide produced by endophytic *Cryptosporiopsis quercina* from *Tripterigium wiflordii*, had antifungal activity against *Candida albicans* (Strobel et al., 1999). A group of peptides, echinocandins isolated from endophytic *Cryptosporiopsis* spp. and *Pezicula* spp. in *Pinus sylvestris* and *Fagus sylvatica*, were antimicrobial (Noble, Langley, Sidebottom, Lane, & Fisher, 1991). These antimicrobial peptides isolated from eukaryotes are highly cytotoxicity; this currently makes them undesirable for use in foods, but they present a great opportunity to find reliable and novel antimicrobial natural products (Yu et al., 2010).

10.5 Commercial natural antimicrobials

Antimicrobial technologies, such as high pressure, pulsed light, ozone generators, ultrasound and UV systems, are widely available and can be ordered to accommodate processing lines, production volumes and specific commodity requirements. Several commercial products are available based on the action of natural antagonists, including *Pseudomonas syringae*, *Candida oleofila* or *Cryptococcus albidus*—all for the control of disease after harvest from Bio-Coat and Biocure. Cellulose is used in several commercial coatings supplied by Mantrose Bradshaw Zinsser Group and Surface Systems International Ltd. Domca produces a formula based on active organosulfur compounds from *Alliaceae* plants, natural organic acids and inert excipients for disease control in fruit after harvest. Several coating products based on chitosan supplied by NovaChem, Courtaulds Group, Pace International, Shield-Brite, Agri-Tech Inc. and others are currently available in the market. There is a large range of commercial antimicrobial packaging available for various food applications from various suppliers including Curwood, Hercules Chemical Co. and Natural Pak Produce.

10.6 Conclusion and future trends

None of the chemical treatments currently available are able to achieve more than two to three logarithmic unit reductions in numbers of potentially pathogenic

micro-organisms present on products. The combination of technologies is referred to as hurdle technology (Goodburn & Wallace, 2012). Hurdle technology, which involves simultaneous multiple preservation approaches, has generally met with success in controlling pathogens and maintaining food quality during storage (Holley & Patel, 2005; Leistner, 2000), yet food safety issues remain. Applying a combination of two or more chemical and/or physical treatments may increase the effectiveness of their antimicrobial function through synergistic interactions between them.

The fact that food matrices usually are more complex and challenging than laboratory media needs to be borne in mind. Many foods consist of a water phase and a lipid phase. This must be considered when deciding how to apply the hurdles and distribute them in the foods. It is important to remember that using hurdles is a delicate balance between inhibiting the growth of spoilage organisms and pathogenic bacteria and at the same time maintaining a satisfactory quality of the food when consumed.

Current active topics in research for use as antimicrobials and preservation for food systems include microencapsulation, nanotechnology, contact-free decontamination and quorum sensing. Currently, there is a large array of natural technologies and antimicrobial agents actively being researched. However, individually, none has yet proven to be as reliable, have as broad a spectrum of activity, effectiveness, ease of use and low cost and be as all around efficient as traditional chemical agents. The solution to this is combining a number of preservatives, making use of the synergistic hurdle concept. Depending on the food, different combinations of different hurdles must be combined and optimised. Whatever the natural antimicrobial method chosen, selection must be based on sensory and chemical compatibility with the target food, its stability given the type of primary preservation system used, its effectiveness against the expected undesirable micro-organisms, its safety and its cost effectiveness (Xi et al., 2011).

References

Abriouel, H., Benomar, N., Lucas, R., & Gálvez, A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. *International Journal of Food Microbiology*, *144*, 487−496.

Ackermann, H. W., & Kropinsk, A. M. (2007). Curated list of prokaryote viruses with fully sequenced genomes. *Research in Microbiology*, *158*, 555−566.

Aguiló-Aguayo, I., Charles, F., Renard, C. M. G. C., Page, D., & Carlin, F. (2013). Pulsed light effects on surface decontamination, physical qualities and nutritional composition of tomato fruit. *Postharvest Biology and Technology*, *86*, 29−36.

Ahmed, L., Martin-Diana, A. B., Rico, D., & Barry-Ryan, C. (2011a). Extending the shelf − life of tomato using by-product from cheese industry. *Journal of Food Processing and Preservation*, *36*, 141−151.

Ahmed, L., Martin-Diana, A. B., Rico, D., & Barry-Ryan, C. (2011b). Quality and nutritional status of fresh-cut tomato as affected by spraying of delactosed whey permeate compared to industrial washing treatment. *Food and Bioprocess Technology*, *5*, 3103−3114.

Ahmed, L., Martin-Diana, A., Rico, D., & Barry-Ryan, C. (2013). Effect of delactosed whey permeate treatment on physico-chemical, sensorial, nutritional and microbial properties of

whole tomatoes during postharvest storage. *LWT − Food Science and Technology*, *51*, 367−374.
Anino, S. V., Salvatori, D. M., & Alzamora, S. M. (2006). Changes in calcium level and mechanical properties of apple tissue due to impregnation with calcium salts. *Food Research International*, *39*, 154−164.
Aung, M. M., & Chang, Y. S. (2014). Traceability in a food supply chain: safety and quality perspectives. *Food Control*, *39*, 172−184.
Barbut, S. (2014). Review: automation and meat quality-global challenges. *Meat Science*, *96*, 335−345.
Barry-Ryan, C. (2012). Chapter 20 − The use of mild heat treatment for fruit and vegetable processing. In Gómez-López (Ed.), *Decontamination of fresh and minimally processed products*. Wiley-Blackwell Publishers.
Bintsis, T., Litopoulou-Tzanetaki, E., & Robinson, R. K. (2000). Existing and potential applications of ultraviolet light in the food industry a critical review. *Journal of the Science of Food and Agriculture*, *80*, 637−645.
Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., et al. (2012). Food fermentations: microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*, 87−97.
Brody, A. L., Strupinsky, E. R., & Kline, L. R. (2001). *Active packaging for food applications*. Lancaster: Technomic Publishing.
Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods: a review. *International Journal of Food Microbiology*, *94*, 223−253.
Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*, 131−149.
Chemat, F., Zill-e-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrasonics Sonochemistry*, *18*, 813−835.
Claussen, I. C. (2011). Superchilling concepts enabling safe, high quality and long term storage of foods. *Procedia Food Science*, *1*, 1907−1909.
Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*, 1−20.
Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*, 308−316.
Durango, A. M., Soares, N. F. F., & Andrade, N. J. (2006). Microbiological evaluation of an edible antimicrobial coating on minimally processed carrots. *Food Control*, *17*, 336−341.
Eklund, M. W., Poysky, F. T., & Habig, W. H. (1989). Chapter 2 − Bacteriophages and plasmids in *Clostridium botulinum* and *Clostridium tetani* and their relationship to production of toxins. In L. L. Simpson (Ed.), *Botulinum Neurotoxin and Tetanus Toxin* (pp. 25−51). San Diego, CA: Academic Press.
Fitzgerald, R. J., & Murray, B. A. (2006). Bioactive peptides and lactic fermentations. *International Journal of Dairy Technology*, *59*, 118−125.
Gaggia, F., Di Gioia, D., Baffoni, L., & Biavati, B. (2011). The role of protective and probiotic cultures in food and feed and their impact on food safety. *Trends in Food Science and Technology*, *22*, S58−S66.
Gómez-López, V. M., Ragaert, P., Ryckeboer, J., Jeyachchandran, V., Debevere, J., & Devlieghere, F. (2007). Shelf-life of minimally processed cabbage treated with neutral electrolysed oxidising water and stored under equilibrium modified atmosphere. *International Journal of Food Microbiology*, *117*, 91−98.
Goodburn, C., & Wallace, C. (2012). The microbiological efficacy of decontamination methodologies for fresh produce: a review. *Food Control*, *32*, 418−427.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The anti-microbial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, *124*, 91−97.

Gutierrez, J., Rodriguez, G., Barry-Ryan, C., & Bourke, P. (2008). Efficacy of plant essential oils against food-borne pathogens and spoilage bacteria associated with ready to eat vegetables: antimicrobial and sensory screening. *Journal of Food Protection*, *71*, 1846−1854.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and their plant extracts. *Journal of Applied Microbiology*, *86*, 985−990.

Hardisson, A., Rubio, C., Frıas, I., Rodrıguez, I., & Reguera, J. I. (2002). Content of sulphite in frozen prawns and shrimps. *Food Control*, *13*, 275−279.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*, *22*, 273−292.

Issa-Zacharia, A., Kamitani, Y., Miwa, N., Muhimbula, H., & Iwasaki, K. (2011). Application of slightly acidic electrolyzed water as a potential non-thermal food sanitizer for decontamination of fresh ready-to-eat vegetables and sprout. *Food Control*, *22*, 601−607.

James, S. J., & James, C. (2010). The food cold-chain and climate change. *Food Research International*, *43*, 1944−1956.

Jurado-Alameda, E., García-Román, M., Altmajer-Vaz, D., & Jiménez-Pérez, J. L. (2012). Assessment of the use of ozone for cleaning fatty soils in the food industry. *Journal of Food Engineering*, *110*, 44−52.

Karbowiak, T., Debeaufort, F., Voilley, A., & Trystram, G. (2009). From macroscopic to molecular scale investigations of mass transfer of small molecules through edible packaging applied at interfaces of multiphase food products. *Innovative Food Science and Emerging Technologies*, *10*, 116−127.

Kashif, M., Khan, I., Schutyser, M. A. I., Schroën, K., & Boom, R. M. (2012). Electrostatic powder coating of foods − state of the art and opportunities. *Journal of Food Engineering*, *111*, 1−5.

Keskinen, L. A., Burke, A., & Annous, B. A. (2009). Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. *International Journal of Food Microbiology*, *132*, 134−140.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. *International Journal of Food Microbiology*, *144*, 51−63.

Korhonen, H. (2009). Milk-derived bioactive peptides: from science to applications. *Journal of Functional Foods*, *1*, 177−187.

Kozempel, M., Radewonuk, E. R., Scullen, O. J., & Goldberg, N. (2002). Application of the vacuum/steam/vacuum surface intervention process to reduce bacteria on the surface of fruits and vegetables. *Innovative Food Science & Emerging Technologies*, *3*, 63−72.

Leistner, I. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, *55*, 181−186.

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., et al. (2011). Microbial control of food-related surfaces: Na-chlorophyllin-based photosensitization. *Journal of Photochemistry and Photobiology B: Biology*, *105*, 69−74.

Luksiene, Z., & Paskeviciute, E. (2011). Microbial control of food-related surfaces: Na-Chlorophyllin-based photosensitization. *Journal of Photochemistry and Photobiology B: Biology*, *105*, 69−74.

Marra, F., Zhang, L., & Lyng, J. G. (2009). Radio frequency treatment of foods: review of recent advances. *Journal of Food Engineering*, *91*, 497−508.

Martin-Diana, A. B., Rico, D., Barry-Ryan, C., Frias, J. M., Mulcahy, J., & Henehan, G. T. M. (2005). Comparison of calcium lactate with chlorine as a washing treatment for fresh-cut lettuce and carrots: quality and nutritional parameters. *Journal of the Science of Food and Agriculture*, *85*, 2260−2268.

Martín-Diana, A. B., Rico, D., Frías, J., Henehan, G. T. M., Mulcahy, J., Barat, J. M., et al. (2006a). Effect of calcium lactate and heat-shock on texture in fresh-cut lettuce during storage. *Journal of Food Engineering*, *77*, 1069−1077.

Martín-Diana, A. B., Rico, D., Mulcahy, J., Frías, J., Henehan, G. T. M., & Barry-Ryan, C. (2006b). Whey permeate as bio-preservative for shelf-life maintenance of fresh-cut vegetables. *Innovative Food Science & Technologies*, *7*, 112−123.

Mikani, A., Etebarian, H. R., Sholberg, P. L., O'Gorman, D. T., Stokes, S., & Alizadeh, A. (2008). Biological control of apple gray mold caused by Botrytis mali with Pseudomonas fluorescens strains. *Postharvest Biology and Technology*, *48*, 107−112.

Muzzarelli, C., & Muzzarelli, R. A. (2002). Natural and artificial chitosan inorganic composites. *Journal of Inorganic Biochemistry*, *92*, 89−94.

Noble, H. M., Langley, D., Sidebottom, P. J., Lane, S. J., & Fisher, P. J. (1991). An echinocandin from an endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus sylvatica*. *Mycological Research*, *95*, 1439−1440.

O'Donnell, C. P., Tiwari, B. K., Bourke, P., & Cullen, P. J. (2010). Effect of ultrasonic processing on food enzymes of industrial importance. *Trends in Food Science and Technology*, *21*, 358−367.

Ohlsson, T. (2002). Introduction. In T. Ohlsson, & N. Bengtsson (Eds.), *Minimal processing technologies in the food industry*. Cambridge, UK: Woodhead Publishing.

Oliveira, M., Viñas, I., Colàs, P., Anguera, M., Usall, J., & Abadias, M. (2014). Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. *Food Microbiology*, *38*, 137−142.

Oussalah, M., Caillet, S., Saucier, L., & Lacroix, M. (2006). Antimicrobial effects of selected plant essential oils on the growth of a Pseudomonas putida strain isolated from meat. *Meat Science*, *73*, 236−244.

Palou, E., Lopez-Malo, A., Barbosa-Canovas, G. V., & Welti-Chanes, J. (2000). High hydrostatic pressure and minimal processing. In S. M. Alzamora, M. S. Tapia, & A. Lopez-Malo (Eds.), *Minimally processed fruits and vegetables*. Maryland: Aspen.

Pao, S., Kelsey, D. F., & Long, W., III (2009). Spray washing of tomatoes with chlorine dioxide to minimize Salmonella on inoculated fruit surfaces and cross contamination from revolving brushes. *Journal of Food Protection*, *12*, 2448−2452.

Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garret, E. H., Farber, J. N., et al. (2003). Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, *2*, 161−173.

Pipek, P., Houska, M., Jelenı kova, J., Kyhos, K., Hoke, K., & Sikulova, M. (2005). Microbial decontamination of beef carcasses by combination of steaming and lactic acid spray. *Journal of Food Engineering*, *67*, 309−315.

Pipek, P., Sikulova, M., Jelenıkova, J., & Izumimoto, M. (2005). Colour changes after carcasses decontamination by steam and lactic acid. *Meat Science*, *69*, 673−680.

Rajkovic, A., Smigic, N., & Devlieghere, F. (2010). Contemporary strategies in combating microbial contamination in food chain. *International Journal of Food Microbiology*, *141*, S29−S42.

Rico, D., Martın-Diana, A. B., Barat, J. M., & Barry-Ryan, C. (2007). Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science and Technology*, *18*, 373−386.

Rico, D., Martin-Diana, A. B., Barry-Ryan, C., Frias, J. M., Henehan, G. T. M., & Barat, J. M. (2008). Use of neutral electrolysed water (EW) for quality maintenance and shelf life extension of minimally processed lettuce. *Innovative Food Science and Technologies*, *9*, 37−48.

Rojas-Grau, M. A., Soliva-Fortuny, R., & Martın-Belloso, O. (2009). Edible coatings to incorporate active ingredients to freshcut fruits: a review. *Trends in Food Science & Technology*, *20*, 438−447.

Saftner, R. A., Baj, J., Abbott, J. A., & Lee, Y. S. (2003). Sanitary dips with calcium propionate, calcium chloride, or calcium amino acid chelates maintain quality and shelf stability of fresh-cut honeydew chunks. *Postharvest Biology and Technology*, *29*, 257−269.

Sapers, G. M. (2009). Chapter 16 − Disinfection of contaminated produce with conventional washing and sanitizing technology. *The Produce Contamination Problem* (pp. 393−424). USA: Academic Press.

Sebranek, J. G., & Bacus, J. N. (2007). Cured meat products without direct addition of nitrate or nitrite: what are the issues? *Meat Science*, *77*, 136−147.

Smilanick, S. L., Crisosto, C., & Mlikota, F. (1999). Postharvest use of ozone on fresh fruit. *Perishables Handling Quarterly Issue*, *99*, 10−14.

Song, J. Y., An, G. H., & Kim, C. J. (2003). Color, texture, nutrient contents, and sensory values of vegetable soybeans [Glycine max (L.) Merrill] as affected by blanching. *Food Chemistry*, *83*, 69−74.

Strobel, G. A., Miller, R. V., Martinez-Miller, C., Condron, M. M., Teplow, D. B., & Hess, W. M. (1999). Cryptocandin a potentand antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. *Microbiology*, *145*, 1919−1926.

Strobel, G. A., Torzynski, R., & Bollon, A. (1997). *Acremonium* sp.a leucinostatin A producing endophyte of European yew (*Taxus baccata*). *Plant Science*, *128*, 97−108.

Sunen, E., Fernandez-Galian, B., & Aristimuno, C. (2001). Antimicrobial activity of wood smoke condensates against *Aeromonas hydrophila*, *Yersinia enterocolitica* and *Listeria monocytogenes* at low temperature. *Food Microbiology*, *18*, 387−393.

Vega-Mercado, H., Martín-Belloso, O., Qin, B. L., Chang, F. J., Góngora-Nieto, M. M., Barbosa-Cánovas, G. V., et al. (1997). Non-thermal food preservation: pulsed electric fields. *Trends in Food Science & Technology*, *8*, 151−157.

Vitt, S. M., Himelbloom, B. H., & Crapo, C. A. (2001). Inhibition of Listeria inocula and *Listeria monocytogenes* in a laboratory medium and cold-smoked salmon containing liquid smoke. *Journal of Food Safety*, *2*, 111−125.

Xi, Y., Sullivan, G. A., Jackson, A. L., Zhou, G. H., & Sebranek, J. G. (2011). Use of natural antimicrobials to improve the control of *Listeria monocytogenes* in a cured cooked meat model system. *Meat Science*, *88*, 503−511.

Ye, J. X., Kostrzynka, M., Dunfield, K., & Warriner, K. (2009). Evaluation of a biocontrol preparation consisting of *Enterobacter asburiae* JX1 and a lytic bacteriophage cocktail to suppress the growth of *Salmonella* Javiana associated with tomatoes. *Journal of Food Protection*, *72*, 2284−2292.

Ye, J. X., Kostrzynka, M., Dunfield, K., & Warriner, K. (2010). Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *Journal of Food Protection*, *73*, 9−17.

Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., et al. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiological Research*, *165*, 437−449.

Nanostructured and nanoencapsulated natural antimicrobials for use in food products

11

A. Brandelli[1], *T.M. Taylor*[2]
[1]Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [2]Texas A&M University, College Station, TX, USA

11.1 Introduction

Food safety is a topic of major concern in the current development of new food products and food processing techniques. Despite advances in technology, the food industry is constantly challenged to reduce the economical losses caused by food spoilage and to avoid the transmission of microbial pathogens along the food chain. Strategies to control food safety hazards must consider the emergence of new pathogens and microorganisms that have the ability to adapt to new methods of food processing, packaging, and storage. This concern runs in parallel with the growing demands of the consumers for "more natural" foods and ready-to-eat products that should be nutrient wealthy and microbiologically safe as well. Thermal treatment has been traditionally used as a major food preservation technology, but emerging technologies such as pulsed electric field and high hydrostatic pressure have been proposed with the aim of producing more "fresh-tasting" product with the guarantee of food safety. Among the proposed new technologies, the use of natural antimicrobial compounds is a field that has gained relevance, because these substances would not cause any toxic or undesirable effect to the consumer. Despite the advances in and successful examples of the use of natural antimicrobials in food, some promising antimicrobials may have an impaired effect *in situ* due to undesirable interactions and inactivation in the food matrix (Cleveland, Montville, Nes, & Chikindas, 2001; Gálvez, Abriouel, López, & Omar, 2007).

Nanotechnology proposes the use and/or development of materials in which at least one dimension is less than 100 nm. The advances in materials science and analytical methodologies led to a rapid development of nanotechnology. This impressive advance has had a strong impact in the fields of biomedical sciences and biotechnology, and numerous nanometric systems have been developed as effective carriers for bioactive substances. Delivery and controlled release of several drugs can be strongly improved with the use of nanoparticulate systems such as polymeric nanoparticles and nanofibers, which can allow effective drug accumulation into target cells (Emerich & Thanos, 2006; Heunis & Dicks, 2010).

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Nanotechnology can be also valuable in food preservation. Some advantages of nanotechnology would be the protection of the antimicrobial against premature inactivation in the food matrix and controlled release of the substance, allowing a putative increase in the shelf life. Thus, nanobiotechnology has enormous potential for improvement of food safety as a powerful tool for delivery and controlled release of natural antimicrobials.

11.2 Natural food antimicrobials

A number of antimicrobial substances are of natural occurrence in foods. Some of these substances have been described in detail in other chapters within this book. In this chapter, a brief description of some natural antimicrobials and their use in nanotechnological approaches is presented. These include lysozyme, lactoferrin, bacteriocins, and some plant-derived antimicrobial substances.

Lysozyme is present as a major protein in egg white and is used as an antimicrobial in food formulations. This enzyme is also present in milk, which contains other well-known natural antimicrobial substances such as lactoferrin and lactolipids. The application of lysozyme as an antimicrobial in several food products is widely reported, including raw and processed meat and milk, fish products, cheese, and some beverages (Juneja, Dwivedi, & Yan, 2012). Lysozyme is a bacteriolytic enzyme that has the ability to hydrolyze the β-1,4 linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of bacterial cell walls (Masschalck & Michiels, 2003). Peptidoglycan is a major component of the cell wall of Gram-positive bacteria, which makes them susceptible to the activity of lysozyme. Lysozyme has been reported as a strong natural preservative, resulting in extended shelf life of food products and inhibition of the growth of pathogenic bacteria such as *Clostridium* spp., *Listeria monocytogenes*, *Bacillus* spp., *Escherichia coli*, and *Pseudomonas* spp. (Davidson, Critzer, & Taylor, 2013).

Lactoferrin is an antimicrobial glycoprotein of milk origin that shows iron-binding capability. Lactoferrin presents a broad spectrum of antimicrobial activity, including a wide range of bacteria and viruses (Lönnerdal & Iyer, 1995). Other biological activities have been associated with lactoferrin and its derived peptides, such as regulation of immune function, stimulation of intestinal proliferation and differentiation, and facilitation of iron absorption (Legrand & Mazurier, 2010; Sinha, Kayshik, Kaur, Sharma, & Singh, 2013). Lactoferrin binds to specific targets on the cell surface of Gram-negative bacteria, causing injury to the outer membrane. Thus, the antimicrobial activity of lactoferrin is attributed to its protein structural conformation, which is essential to warrant a tight binding to the bacterial cell surface (Lönnerdal & Iyer, 1995; Sinha et al., 2013). This antimicrobial protein has been applied in a variety of commercial products, including meat products, human infant formulas, and sports beverages, and as a dietary supplement with claims of improved immune system function (Juneja et al., 2012).

Some antimicrobials that are naturally present in food are produced by the autochthonous microbiota, and possibly the most heavily studied are those produced by

lactic acid bacteria (LAB). A class of natural antimicrobials that are widely produced by LAB are the organic acids. The use of lactic acid fermentation has been successfully used for a long time as a strategy of food preservation (Gibbs, 1987). These substances often have broad inhibitory spectra, but their utility is limited in some foods owing to environmental pH and microbial adaptation or degradation of acidic species (Doores, 2005).

Many antimicrobial peptides have been proposed as natural food preservatives. Among them, several bacteriocins have been described with potential for application as natural food preservatives. Bacteriocins are produced by several bacteria, but extensive investigation has been devoted to those produced by LAB, because these bacteria are often considered safe for food use. Bacteriocins produced by LAB have several interesting characteristics that make them suitable for food preservation. Some attractive properties often associated with LAB bacteriocins include: (1) they are generally recognized as safe substances (GRAS); (2) they are not active and are nontoxic on eukaryotic cells; (3) they are inactivated by digestive proteases, such as trypsin, having little influence on gut microbiota; (4) they are usually heat and low pH tolerant; (5) they have a relatively broad antimicrobial spectrum against many common foodborne pathogenic and spoilage bacteria; (6) they show a bactericidal mode of action; and (7) they show no cross-resistance with antibiotics. In addition, their genetic determinants are usually plasmid encoded, facilitating genetic manipulation (Gálvez et al., 2007).

Among LAB bacteriocins, the lantibiotic nisin and class IIa bacteriocins (pediocin-like bacteriocins) have been the subject of exhaustive investigation (Breukink & Krujiff, 1999; Drider, Fimland, Héchard, McMullen, & Prévost, 2006; McAuliffe, Ross, & Hill, 2001; Nagao et al., 2006). Commercial preparations of nisin and pediocin are currently available, but other bacteriocins such as lacticin 3147 and enterocin AS-48 offer promising perspectives as food preservatives (Gálvez et al., 2007). Nisin is produced by *Lactococcus lactis* subsp. *lactis* and has been approved for food use in several countries. This bacteriocin is formed by 34 amino acid residues and shows antimicrobial activity against Gram-positive bacteria in the nanomolar range. Nisin contains dehydrated residues and five lanthionine rings, which impose structural constraints on the peptide. In addition to nisin A and Z, the most common forms of this bacteriocin, subtypes U, Q, and F are also described (Piper, Hill, Cotter, & Ross, 2011). Nisin primarily binds to the lipid II, which is an essential intermediate in cell wall biosynthesis, and subsequently forms pores in the cell membrane of the target bacterium. Because of its GRAS status and its activity against important food pathogens, including *Staphylococcus aureus*, *Clostridium* spp., and *L. monocytogenes*, nisin is an interesting antimicrobial to be investigated for nanotechnology-based strategies for food preservation (Malheiros, Daroit, & Brandelli, 2010).

Pediocin is also a well-studied bacteriocin that belongs to class IIa, also called antilisterial bacteriocins, because of its prominent antimicrobial activity against *Listeria* spp. Pediocin PA-1/AcH is produced by strains of *Pediococcus acidilactici* and was one of the first bacteriocins IIa to be characterized. This bacteriocin induces leakage of K^+ and amino acids from target cells, inducing a rapid depletion of intracellular

ATP in susceptible bacteria (Drider et al., 2006). Despite pediocin being quite active against *Listeria*, other Gram-positive bacteria belonging to the genera *Enterococcus*, *Lactobacillus*, and *Clostridium* are also inhibited by this bacteriocin (Eijsink, Skeie, Middelhoven, Bruberg, & Nes, 1998).

A number of antimicrobial substances derived from various plant parts have been described, and many of these substances can be useful preservatives for food use. In particular, essential oils contain active ingredients showing broad antibacterial and antifungal activities (Davidson et al., 2013; Naidu & Davidson, 2000). Most essential oils from herbs and spices are considered as GRAS substances. However, their typical flavors represent a major constraint to their use as food preservatives, because the amount needed for an effective antimicrobial activity in foods often exceeds their organoleptic acceptance threshold. The most important active compounds that make essential oils effective as antimicrobial agents include saponins, phenolic compounds such as flavonoids, terpenes, and their precursors (Juneja et al., 2012).

Phytophenols are widely distributed in plants, and essential oils from sage, cloves, rosemary, and other spices contain active compounds such as thymol, eugenol, cinnamic acid, benzoic acid, coumarin, and terpene (Naidu & Davidson, 2000). Cinnamon and clove essential oils are rich in cinnamic aldehyde and eugenol, respectively, exhibiting a broad spectrum of antibacterial activity, such as against *Campylobacter jejuni*, *L. monocytogenes*, and *Salmonella* serovar Enteritidis. Thymol and carvacrol have antimicrobial activity against a broad range of food-borne pathogens, such as *L. monocytogenes*, *Salmonella* spp., *S. aureus*, and *Yersinia enterocolitica* (Davidson et al., 2013).

Saponins are naturally occurring glycosides in many plants, such as *Solanum* and *Allium* spp., clover, and a variety of herbs and seeds (Cowan, 1999). Saponins have activity against a wide range of microorganisms, including bacteria such as *S. aureus*, *Pseudomonas fluorescens*, *E. coli*, and *Salmonella* serovar Typhimurium, and fungi such as *Aspergillus* spp. (Naidu & Davidson, 2000). Saponins interact with sterols and fatty acids on microbial membranes, and their antifungal activity depends on the specific structural features of their aglycone structure.

A number of plant antimicrobial compounds are naturally produced as secondary metabolites or are generated by plant enzymes on inactive precursors to enhance the plant defense system under adverse conditions. In garlic, mustard, and horseradish, the enzyme myrosinase converts glucosinolates into diverse isothiocyanates, some of them showing important antimicrobial activity. Allicin is an active compound of fresh crushed garlic that presents a wide range of antimicrobial activities, including against bacteria, fungi, viruses, and human intestinal protozoan parasites (Ankri & Mirelman, 1999). A broad range of bacteria and yeast are also inhibited by allyl isothiocyanate generated from the hydrolysis of sinigrin, a thioglycoside found in the family Cruciferae (Shofran, Purrington, Breidt, & Fleming, 1998). These natural antimicrobials have been incorporated in nanostructures aiming toward the improvement of their antimicrobial capabilities for food preservation. Examples of these nanosystems and their potential food applications are further discussed in this chapter.

11.3 Nanostructures for antimicrobial delivery

The development of nanostructures for drug delivery and controlled release has been a point of intensive investigation. Diverse antimicrobials, including conventional antibiotics and natural antimicrobial substances, have been incorporated in nanostructures (Brandelli, 2012). The most important nanostructures that can be used for delivery of antimicrobial substances are described here and summarized in Table 11.1.

11.3.1 Nanoencapsulation

The encapsulation of antimicrobial substances into nanoparticles may increase their stability and efficacy in practical applications. Nanoparticles are colloidal systems assembled by lipids (liposomes) or polymers. The use of biodegradable polymers as constituent material for nanoparticles has attracted more attention because of their increased stability in biological fluids during storage in comparison to liposomes. Nanoparticles are spheric delivery systems with a nanometric diameter and they comprise nanocapsules (or nanovesicles) and nanospheres, which differ in composition and structural organization (Figure 11.1). Nanovesicles are formed by a polymeric membrane that encloses an oily core in which the antimicrobial is confined. Alternatively, the antimicrobial can be adsorbed to the polymeric coat. Otherwise, the nanospheres are formed by a polymeric matrix-type structure. In this case, the antimicrobial can be retained or adsorbed to the structure (Brandelli, 2012; Vauthier & Bouchemal, 2009).

Nanoliposomes are a particular type of nanovesicle that have an internal aqueous core rounded by a self-assembled coat of amphiphilic lipids. As the liposomes contain both lipid and aqueous phases, they can be used for the entrapment and delivery of hydrophilic, hydrophobic, and amphiphilic molecules. A major challenge in producing nanoliposomes is to accomplish the formation of vesicles with the right size, polydispersity, structure, and encapsulation efficiency (Jesorka & Orwar, 2008; Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008).

A diversity of nanoparticles has been developed, and these nanoparticulate systems are generally designed for therapeutical applications, most of them aimed at the targeting of anticancer and antimicrobial drugs (Brannon-Peppas & Blanchette, 2012). Several methods are described for the preparation of polymeric nanoparticles, which can be usually classified into methods based on *in situ* polymerization of dispersed monomers or precipitation of preformed polymers, such as polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), and poly-ε-caprolactam (PCL), among other polymers.

The polymerization of alkyl cyanoacrylates in emulsion leads to matricial nanoparticles called nanospheres, whereas in the interfacial polymerization method, the addition of an organic solvent and oil in this medium provides vesicular nanostructures, the nanocapsules (Vauthier & Bouchemal, 2009). In the solvent evaporation method, the polymer along with the drug is dissolved in volatile organic solvent and poured into continuously stirring aqueous phase with or without emulsifier and sonicated (Yadav, Kumari, & Yadav, 2011). Nanoparticles can also be prepared

Table 11.1 Nanostructures with potential application for delivery of natural antimicrobials

Nanostructure	Characteristics	Typical composition
Inorganic nanoparticles	Typically composed of inorganic compounds; they can be used to produce a myriad of nanostructures with varying size, shape, and porosity	Silica, alumina, metals, metal oxides, calcium phosphates
Polymeric nanoparticles	Mostly biodegradable and biocompatible, suitable for the entrapment and delivery of a wide range of therapeutic agents. They have been adopted as a preferred method for nanoparticulate drug delivery	Gelatin, chitosan, poly(lactic-co-glycolic acid) copolymer, polylactic acid, polyglycolic acid, poly(alkylcyanoacrylate), poly(methylmethacrylate), poly(butyl)cyanoacrylate
Solid lipid nanoparticles	Lipid-based colloidal nanocarriers. They have a relatively rigid core consisting of hydrophobic lipids that are solid at room and body temperatures, surrounded by a monolayer of phospholipids	Triglycerides, fatty acids, partial glycerides
Nanoliposomes	Concentric bilayered nanovesicles surrounded by a phospholipid membrane. Their amphiphilic nature, ease of surface modification, and good biocompatibility make them an appealing solution for delivery of proteins and peptides	Phospholipids
Nanoemulsions	Oil-in-water dispersions with droplet diameter in the nanometric range. They are prepared using high shearing forces and are thermodynamically stable	Vegetable oils and phospholipids (emulsifier)
Nanocrystals	Aggregates of molecules that can be combined into a crystalline form of the drug surrounded by a thin coating of surfactant	Poorly water-soluble drugs[a]
Dendrimers	Highly branched structures containing multiple molecular "hooks" on their surfaces that can be used to attach cell-identification tags, enzymes, and other molecules	Poly(amidoamine)

Table 11.1 Continued

Nanostructure	Characteristics	Typical composition
Nanotubes	Self-assembling sheets of atoms or molecules arranged in tubes. They may be organic or inorganic in composition and can be produced as single- or multiwalled structures	Carbon, polymers[b]
Nanofibers	Fibers with diameters less than 100 nm, produced by electrospinning, interfacial polymerization, melt spinning, or electroblowing. They can be modified by surface functionalization, resulting in unique functions of the fiber surface that can be useful for applications as an advanced material	Gelatin, chitosan, poly(lactic-co-glycolic acid), polylactic acid, polyglycolic acid, poly(e-caprolactone), polyethylene oxide

[a]Nanocrystals have other important applications; Cd and Zn selenites are important materials in semiconductor nanotechnology.
[b]Nanotubes of poly(lactic-co-glycolic acid) and self-assembled peptides and proteins are also described.

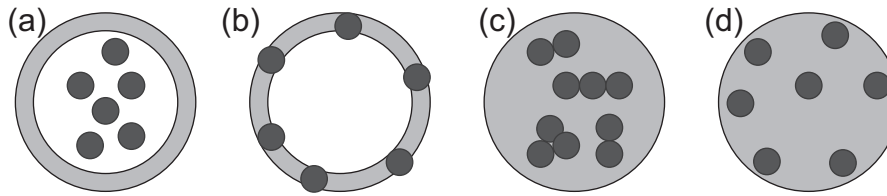

Figure 11.1 Schematic representations of nanovesicles and nanospheres. (a) Antimicrobial substance dispersed in the oil/aqueous core of the nanovesicle; (b) antimicrobial substance embedded in the polymeric wall of the nanovesicle; (c) antimicrobial substance retained in the matrix of the nanosphere; (d) antimicrobial substance adsorbed or molecularly dispersed in the matrix of the nanosphere.

using a solvent extraction method, in which the oil-in-water emulsion is homogenized at high speed, followed by addition of water and solvent evaporation (Guterres, Alves, & Pohlmann, 2007). In addition, nanospheres and nanocapsules can be prepared using preformed polymers by nanoprecipitation or interfacial deposition of polymer, omitting or containing the oil in the formulation, respectively. The method of emulsification−diffusion has been also introduced to obtain nanocapsules, allowing the production of nanospheres by omitting the oil in the formulations (Guterres et al., 2007; Vauthier & Bouchemal, 2009).

Encapsulation of antimicrobial substances into nanoliposomes is mainly reported to be achieved by the thin-film hydration method or reverse-phase method. The thin-film hydration technique consists in a preformed lipid film that is hydrated with an aqueous buffer containing the antimicrobial substance, at a temperature above the main phase-transition temperature of lipids. This process results in a heterogeneous mixture of multilamellar vesicles that can be further processed by membrane extrusion or sonication, resulting in small unilamellar vesicles of a narrower size distribution. In the reversed-phase method, an aqueous solution containing the antimicrobial substance is dropped into the lipid solution to form a water-in-oil emulsion. The emulsion is sonicated yielding a homogeneous opalescent dispersion of reverse micelles. After the organic solvent is evaporated, the resulting highly viscous organogel is reverted to nanovesicles by addition of ultrapure water. These two methodologies were used to encapsulate the antimicrobial peptide nisin into phosphatidylcholine nanovesicles and compared (Malheiros, Micheletto, Silveira, & Brandelli, 2010). The sizes of liposomes prepared by reverse-phase, hydration film using a probe-type and a bath-type ultrasound were 190, 181, and 148 nm, with residual antimicrobial activity, after encapsulation of 25, 50, and 100%, respectively.

Solid lipid nanoparticles (SLNs) are another type of nanostructured system that can be used to deliver antimicrobial substances. These nanoparticles have typical mean diameters in the range of 50−1000 nm and are mainly composed of lipids that are in solid phase at room temperature and surfactants for emulsification. Unlike most polymeric nanoparticle systems, SLN production techniques do not need to employ potentially toxic organic solvents. Also, under optimized conditions they can be produced to incorporate lipophilic or hydrophilic substances and seem to fulfill the requirements for an optimum particulate carrier system (Almeida & Souto, 2007). Solid lipids commonly used in SLNs include fatty acids (e.g., palmitic acid), triglycerides, partial glycerides (e.g., glyceryl monostearate), and waxes. Surfactants such as soybean lecithin, polaxamer 188, and sodium cholate are used as emulsifiers to stabilize lipid dispersions. The typical methods to produce SLNs include spray drying, high-shearing mixing, ultrasound, and high-pressure homogenization (Zhang, Pornpattananangkul, Hu, & Huang, 2010).

Despite the type and the production method, resulting nanoparticles are obtained as aqueous colloidal suspensions. However, during storage, nanoparticle aggregation may occur, resulting in the formation of a precipitate. Also, chemical stability problems related to the polymer or other materials, including the antimicrobial, may occur. The consequence of a limited physicochemical stability during the storage time constitutes a hurdle to the industrial application of aqueous nanoparticles applications. Thus, the development of nanoparticles in solid form could be a solution to minimize the limited stability. The spray drying methodology has been used for dehydration of colloidal systems, particularly liposomes and nanospheres, employing cryoprotective or lyoprotective agents, in general a carbohydrate, to avoid particle aggregation during freezing of the suspensions (Guterres et al., 2007).

As a function of colloidal nature, technical difficulties are found in the physicochemical characterization of nanoparticles. The characterization of suspensions includes their morphological evaluation, particle size distribution, molecular mass

distribution of the polymer, pH, zeta potential, amount of antimicrobial associated with the nanostructure, release kinetics, and stability as a function of storage time and/or environmental condition (e.g., temperature, exposure to light, oxidant, etc.). The data obtained by the characterization of such nanoparticulate systems may lead to the understanding of the nanoparticle organization at molecular level, providing essential information to the proposal of feasible practical applications.

11.3.2 Nanoemulsions

Nanoemulsion (NE) can be defined as a dispersion consisting of oil, surfactant, and an aqueous phase, which is a single optically isotropic and thermodynamically stable liquid solution, usually with a droplet diameter in the range of 10−100 nm.

NEs have much higher surface area and free energy than macroemulsions, which make them effective transport systems. NEs are metastable and can be diluted with water without changing droplet size distribution, showing no problems with inherent creaming, flocculation, coalescence, or sedimentation that are common with macroemulsions. Because NEs are often formulated with compounds that are approved for human consumption (GRAS), they can be taken by the enteric route. Several oils have been used in NE formulations, including castor oil, coconut oil, cotton seed oil, mineral oil, olive oil, soybean oil, and many other vegetable oils. Common emulsifiers are lecithin, PEG−phospholipids, poloxamers, stearylamine, oleylamine, and others. Formulations could also include some additives with the function of tonicity modifiers (glycerol, sorbitol), antioxidants (α-tocopherol, ascorbic acid), and preservatives. In general, NEs are recognized as nontoxic and nonirritating, and thus can be applied to skin and/or mucosal membranes. Otherwise, NEs show poor drug-loading capacity and drug expulsion after polymeric transition during storage, and the NE dispersions have a relatively high water content (Mason, Wilking, Meleson, Chang, & Graves, 2006).

NE preparation by high-pressure homogenization is the method originally used for solid lipid nanoparticles. This technique uses a high-pressure homogenizer to produce NEs with very low particle sizes, up to 1 nm. Another similar method is microfluidization, which uses a high-pressure positive displacement pump that forces the product through the interaction chamber consisting of very small conduits (Shah, Bhalodia, & Shelat, 2010). These high-pressure methods can be used for fabrication of NEs at the laboratory and industrial scales, whereas ultrasonic emulsification is primarily used at the laboratory scale. The phase-inversion temperature method employs temperature-dependent solubility of nonionic surfactants to modify their affinities for water and oil as a function of the temperature. It has been observed that polyethoxylated surfactants tend to become lipophilic on heating owing to dehydration of the polyoxyethylene groups. The fast-cooling dilution process leads to nanosized droplets. This method involves heating of the components and it may be difficult to incorporate thermolabile substances without affecting their stability. Other techniques such as the solvent displacement method and phase inversion composition method have also been described (Lovelyn & Attama, 2011). The major disadvantage of the use of organic solvents is that additional inputs are required for their removal from NE. Although

the process of solvent removal may be simple at the laboratory scale it can cause several difficulties during scale-up. In addition, a high ratio of solvent to oil is required to obtain a NE with a desirable droplet size. NEs obtained from the phase-inversion composition process are not thermodynamically stable, although they might have high kinetic energy and long-term colloidal stability.

The Michigan Nanotechnology Institute developed antimicrobial NEs, which consisted of 200−600-nm oil-in-water droplets stabilized by surfactants and alcohol. This composite material was prepared using GRAS ingredients by high-speed mixer, and additional size reduction was achieved by a high-pressure microfluidizer. The concentrated emulsions diluted in water resulted in a stable final product. The NE showed a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, enveloped viruses, fungi, and spores (Hamouda et al., 1999). When these nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death. The NEs show selective toxicity to microbes at concentrations that are nonirritating to skin or mucous membrane, achieving a level of topical antimicrobial activity that has been previously achieved only by systemic antibiotics (Baker, Hamouda, Shih, & Myc, 2000).

11.3.3 Nanofibers

Nanofibers are produced from polymers through specific methodologies that form filaments that are nanometers in diameter (Figure 11.2). Although several methods have been described as producing nanofibers, electrospinning is the most cost-effective, simple way to produce large amounts of these nanostructures. The basic concept of electrospinning is to employ electrostatic repulsion of charged polymer jets to produce randomly oriented or aligned nanofibers on the collector surface. One electrode is placed in a polymer solution and the other is linked to a collector. The electrically charged polymer forms a Taylor cone at the tip of the needle and is ejected at a specific

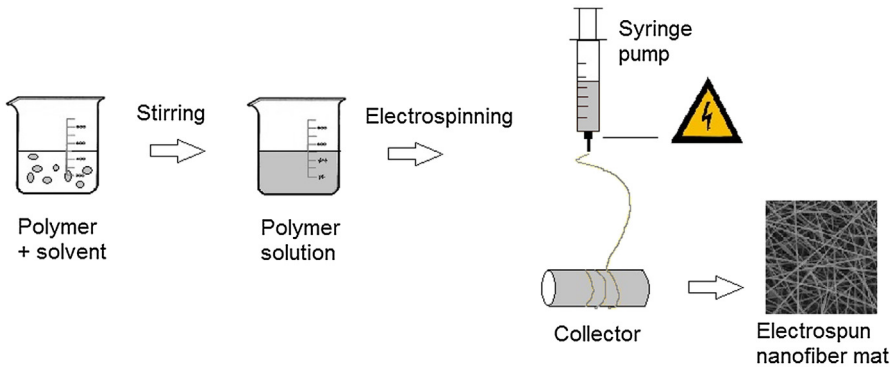

Figure 11.2 Schematic representation of the production of nanofibers by the electrospinning process.

charge. As the polymer solution accelerates, the solvent evaporates, and nanofibers are formed, which are aligned by a rotating collector or an auxiliary electrical field (Teo & Ramakrishna, 2006). Several polymers used to produce spherical nanoparticles, such as PLA, PCL, PLGA, and polyvinylpyrrolidone are also used to produce nanofibers. Also, natural polymers such as gelatin and chitosan may be interesting materials to use to develop nanofibers. The major factors that may influence nanofiber formation by the electrospinning process are temperature, viscosity, conductivity and surface tension of the solution, strength of the electric field, distance between the needle tip and the collector, and humidity (Heunis & Dicks, 2010; Teo & Ramakrishna, 2006). Larger diameter fibers are obtained by increasing the polymer concentration in solution. Also, the morphology of the nanofibers can be altered by the amount of polymer. For example, the increase in PLA concentration from 1% to 3% (w/w) causes the change of a "bead on a string" structure to a nanofiber with a smooth structure (Maretscheck, Greiner, & Kissel, 2008).

Nanofibers have a large surface-to-volume ratio, and manipulation of surface properties may provide these structures a high loading efficiency. Diverse nanofibers have been surface modified with bioactive molecules for advanced biological and therapeutic applications (Yoo, Kim, & Park, 2009). In general, the electrospinning process and the nanofiber morphology are more controllable by using synthetic polymers versus natural polymers. Water-soluble natural polymers are regarded as difficult to process into nanofibers, but considering their unique and specific biological properties, they can be immobilized onto the surface of nanofibers prepared with synthetic polymers. This can allow the development of nanostructures that combine the processing benefits of synthetic polymers with the biological signals on the contacting target cells provided by the natural polymer. Several surface modification techniques for nanofibers have been described, including plasma treatment, wet chemical methods, and graft polymerization. These methods consist in the modification of the nanofiber surface to introduce functional groups (e.g., carboxylate or amines) to improve biocompatibility or to allow the immobilization of bioactive molecules (Wei, Gao, Hou, & Wang, 2005; Yoo et al., 2009). Also, coelectrospinning of the bulk polymer with functional polymer segments or nanoparticles can be useful for the development of nanofibers. This method was used to develop a functionalized polyethylene oxide (PEO) nanofiber, consisting of an anionic antimicrobial peptide terminally conjugated with PEO, which was subjected to coelectrospinning with PEO. The polarizable anionic segment of the antimicrobial peptide influenced the fiber morphology, resulting in the surface orientation of this segment when subjected to the electric field (Sun, Shankar, Borner, Ghosh, & Spontak, 2007). Also, the bacteriocin plantaricin 423 was encapsulated into electrospun PEO nanofibers, maintaining its antimicrobial activity after electrospinning (Heunis, Botes, & Dicks, 2010).

Bioactive molecules can be loaded onto the nanofibers by simple physical adsorption, by assembly of nanoparticles on the nanofiber surface, by multilayer assembly, or through chemical immobilization (Brandelli, 2012; Yoo et al., 2009). The physical immobilization on the surface of nanofibers that have an extremely high surface area-to-volume ratio results in higher drug loading amount per unit of mass. Thus, the release of drugs from a nanofiber surface can enable easy dosage control for

some specific applications such as prophylactic or therapeutic response to bacterial infection. Chemically immobilized molecules are not easily released from the nanofibers, which can be advantageous when the bioactive molecule is introduced to promote cellular recognition or cell adhesive properties. It should be considered that partial inactivation of the biological activity can occur as a consequence of covalent immobilization. However, lysozyme covalently attached to PLGA nanofibers via a PEG linker showed similar activity compared to a native lysozyme (Kim & Park, 2006).

11.3.4 Nanotubes

Carbon nanotubes may be considered as alternative nanostructures for antimicrobial delivery. These nanostructures are cylindrical carbon molecules that present exceptional mechanical and electrical properties, which are useful for nanotechnology. Carbon nanotubes have many structures, differing in length, thickness, number of layers, and diameters often ranging from 1 to 50 nm (Saifuddin, Raziah, & Junizah, 2013).

Carbon nanotubes have a large surface-to-volume ratio and unique electronic properties that make them an interesting material for fabricating new antimicrobial drugs (Aslan et al., 2012; Nepal, Balasubramanian, Simonian, & Davis, 2008). Pristine single-walled carbon nanotubes exhibit an antimicrobial effect in a size-dependent manner, indicating that they might be useful for antimicrobial delivery. The surface modification of carbon nanotubes by chemically attaching organic functional groups helps to produce biocompatible material, improving solubility in physiological solutions and generating sites for the attachment of bioactive molecules. Modification of carbon nanotubes is usually performed by attachment of organic moieties either to carboxylic groups that are formed by oxidation of carbon nanotubes with strong acids or by direct bonding to the surface double bonds (Saifuddin et al., 2013). Composite films with embedded antimicrobial lysostaphin−carbon nanotube conjugates are effective against methicillin-resistant *S. aureus*, reducing viable counts in >99% within 2 h (Pangule et al., 2010). This material may be valuable in preventing the risk of staphylococci infection and biofouling of surfaces.

A concern about the use of carbon nanotubes is related to their potential toxicity, because under certain conditions they can enter human cells and accumulate in the cytoplasm, causing cell death. Although further research is necessary, the available data suggest that under certain conditions, especially those involving chronic exposure, carbon nanotubes can cause a serious risk to human health (Lam, James, McCluskey, Arepalli, & Hunter, 2006).

11.3.5 Self-assembled nanostructures

The spontaneous association of molecules to form three-dimensional structures through a self-assembly process is mediated by weak intermolecular bonds, such as van der Waals bonds, electrostatic interactions, hydrogen bonds, and stacking interactions. This phenomenon is ubiquitous in nature and occurs when lipid molecules form oil droplets in aqueous medium, polypeptides associate to form a functional multimeric protein, or ribosomal proteins and RNA combine into functional ribosomes.

Molecular self-assembly process can be exploited to produce ordered useful nanostructures in various fields, including drug delivery, material science, and microelectronics (Zhang, 2003).

Self-assembled peptides represent many interesting possibilities for application in nanotechnology, including the development of nanostructures for delivery of antimicrobial substances (Brandelli, 2012). Depending on the amino acids that constitute a peptide motif, the peptide building blocks can assemble as nano-ordered structures such as nanotubes or nanospheres. Some cyclic peptides containing alternating even numbers of D- and L-amino acids interact through hydrogen bonding to form an array of self-assembled nanotubes. A family of bola-amphiphiles, linear peptides formed by two hydrophilic sequences linked by a hydrophobic domain, is also described as forming tubular nanostructures (Santoso, Vauthey, & Zhang, 2002). Peptide-based nanotubes can also self-assemble by conjugation of a single hydrophilic peptide motif with a hydrophobic alkyl tail to form a surfactant-like peptide. These amphiphilic peptides can be used as three-dimensional scaffolds because the self-assembled nanofibers form a fibrillar network (Reches & Gazit, 2006). Also, linear peptides with more than 50% charged amino acids with opposing charges are self-assembled into nanofibers that can be used as a scaffold for tissue engineering (Zhang, 2003).

Small dipeptides such as diphenylglycine and diphenylalanine self-assemble into nanotubes at neutral pH and rearrange into spherical structures of about 100 nm in diameter upon dilution. The self-assembly mechanism has been studied, revealing that the peptides initially interact through stacking interactions between aromatic groups to form an extended pleated sheet structure. This structure is stabilized by hydrogen bonds and hydrophobic interaction between aromatic groups. The tubular structures are formed by a closure of the sheet along one axis, or alternatively, a spherical structure may result from a closure of the sheet along two axes (Reches & Gazit, 2006).

Some antimicrobial peptides can form nanostructures. Iturin A is a lipopeptide antibiotic with strong antifungal activity produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens*. This antimicrobial peptide shows a great tendency for self-association into nanovesicles of an average size of 150 nm (Grau, Gómez-Fernández, Peypoux, & Ortiz, 2001). In addition, the bacteriocin lactacin F has been reported to form globular structures with an average diameter from 25 nm to 50 nm, as observed by transmission electron microscopy (Muriana & Klaenhammer, 1991). The purified bacteriocin linocin M18 is a peptide with a molecular mass of 31 kDa. However, this bacteriocin elutes in the void volume of the Superose 6 column (>2000 kDa), and the active fractions from this column revealed particles with a size between 20 nm and 30 nm when observed by electron microscopy (Valdés-Stauber & Scherer, 1994).

Other nanoparticles formed by self-assembled peptide structures show strong antimicrobial activity against diverse bacteria, yeasts, and fungi. These peptide-based nanoparticles have been tested *in vivo* in animal models and they can cross the blood−brain barrier and suppress microbial growth in infected brains, resulting in promising antimicrobial agents for the treatment of brain infections caused by *S. aureus* (Liu et al., 2009) and *Cryptococcus neoformans* (Wang et al., 2010).

11.4 Methods for characterization of nanostructures

The adequate characterization of nanometric structures is essential to establish understanding and control the synthesis and applications of nanomaterials. A variety of techniques can be used to perform the characterization of nanostructures, most of them drawn from materials science. Common techniques are transmission and scanning electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR). The technology for nanoparticle tracking analysis (NTA) provides information on particle size and distribution and a real-time view of nanoparticles in the sample. NTA allows direct tracking of Brownian motion and thus allows the sizing of individual nanoparticles in solution (Walker, 2012). A summary of these methods is displayed in Table 11.2.

11.4.1 Determination of particle size

Considering that particles are three-dimensional objects, a complete description requires three parameters: length, width, and height. Therefore, it is impossible to describe a particle using a single number that equates to particle size. Most sizing techniques assume that the material is spherical because it can be described by a single number, its diameter, thus simplifying the modeling of particle size distributions. It is relevant to consider that different results can be produced when nonspherical particles are measured by different methods.

Table 11.2 Methods for characterization of nanostructures

Method	Information provided	Sensitivity
Dynamic light scattering	Mean particle size and size distribution	1 nm−10 μm
Transmission electron microscopy	Particle size and morphology	<1 nm
Scanning electron microscopy	Particle size and morphology	<1 nm
Atomic force microscopy	Particle size and morphology	1 nm−10 μm
Nanoparticle tracking analysis	Particle size and size distribution	10 nm−1 μm
X-ray diffraction	Mean particle size of bulk sample	<1 nm
Aerosol mass spectroscopy	Particle size and composition	100 nm−3 μm
Zeta potential	Surface charge	
Differential scanning calorimetry	Interactions between drug and nanostructure	
Fourier transform infrared spectroscopy	Interactions between drug and nanostructure	

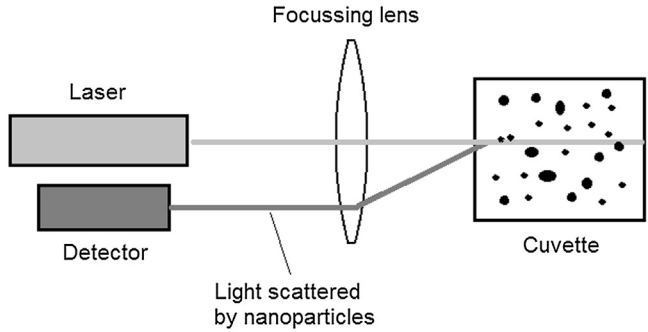

Figure 11.3 Schematic representation of dynamic light scattering method for measurement of nanoparticles.

Particle size of nanoparticles has been generally characterized by DLS, also referred as photon correlation spectroscopy or quasi-elastic light scattering. DLS measures the scattering pattern produced when light is passed though a sample (Figure 11.3). The technique combines measurement with calculations of the diffusion caused by Brownian motion in the sample by a relationship described in the Stokes−Einstein equation. The procedure gives the radius of a particle and therefore an estimation of the average particle size and distribution through the sample (Ruf, 1993). This technique is noninvasive and sensitive enough to determine the size of materials in the submicrometer region, and the results are expressed as the particle hydrodynamic diameter. DLS is an accurate, reliable, and repeatable technique, and the range of particle sizes that can be measured by DLS has been estimated at between 1 nm and 10 μm. Samples for DLS analysis must be a very dilute liquid (solution or suspension) to avoid imprecise scattering of light. The method is sensitive to impurities and the viscosity of the sample must be known. An extension of this technique for higher concentration or opaque samples, such as emulsions, is photon cross-correlation spectroscopy (Pusey, 1999).

Although not as widely used as DLS, acoustic spectroscopy is a method that employs ultrasound, instead of using light, for collecting information on the particles that are dispersed in fluid. This can be done because dispersed particles absorb and scatter acoustic waves similar to light. Acoustic spectroscopy can be used to measure particle size distribution for any particle in a fluid system and measurements can be performed at very high particle concentrations (Povey, 2013). Taylor, Davidson, Bruce, and Weiss (2005) reported the main phase-transition temperature of phosphatidylcholine and phosphatidylcholine/phosphatidylglycerol liposomes containing nisin using acoustic spectroscopy. Inclusion of nisin at 5.0 or 10.0 μg/ml induced slight upshifts in phase transitions, identified by acoustic spectroscopy and differential calorimetry alike.

11.4.2 Microscopy methods

Microscopy methods can be powerful techniques to characterize nanostructures. In addition to the visualization of particle size, this technique allows one to access

morphological properties of the nanostructured material. Thus, many relevant characteristics such as surface and structural irregularities can be accessed.

TEM uses an electron beam, which is transmitted through an ultrathin sample, interacting with the sample as it passes through. An image on a photographic plate or high-quality camera is formed from the interaction of the electrons transmitted through the sample. The samples must therefore be capable of resisting the electron beam and also the high vacuum chamber where the sample is placed (Egerton, 2005). Difficulties are often associated with TEM; the sample preparation can be complicated, because a thin sample on a support grid must be prepared, which, along with the cost and time demands, is a major criticism of TEM. The use of high-resolution TEM may be beneficial when studying samples in the nanoscale range, because it looks at the interference of the electron beam by the sample rather than the absorbance of the beam, as with conventional TEM. However, an adequate interpretation of the results generated by high-resolution TEM requires understanding of the sample because the resulting phase-contrast information can be difficult to interpret. This difficulty may represent a restriction to the use of this technique. Environmental TEM can be carried out *in situ* by using the appropriate gaseous atmosphere as opposed to a vacuum for TEM (Yao & Wang, 2005).

SEM also uses a high-energy electron beam, but in this case the electrons interact with atoms over the surface of the sample, and the resulting signals can be detected to obtain information about the surface topography and composition. The types of signals produced by SEM include secondary electrons, back-scattered electrons, characteristic X-rays, light, specimen current, and transmitted electrons. The most frequent imaging mode collects low-energy secondary electrons, and secondary electron detectors are standard in all scanning electron microscopes. Very high resolution images of a sample surface can be achieved by SEM, revealing details less than 1 nm in size (Egerton, 2005). A requirement of SEM is that the sample must be electrically conductive at the surface, which can be achieved by sputter coating a nonconductive sample. This restrictive condition and the fact that the method can be time-consuming and expensive are the major drawbacks of SEM. Samples can be observed in high vacuum or low vacuum, and in environmental SEM the sample can be observed in a low-pressure gas environment under wet conditions (Yao & Wang, 2005).

Another valuable microscopic technique for nanotechnology is AFM. The technique is based on the use of a mechanical probe to sense the surface of a sample (Yao & Wang, 2005). A cantilever with a nanoscale probe is moved over the surface of a sample and the force between the probe tip and the sample is measured from the deflection of the cantilever (Figure 11.4). The deflection moves a laser spot that reflects onto an arrangement of photodiodes, offering a tridimensional visualization. The cantilever is usually silicon or silicon nitride with a tip radius of curvature on the order of nanometers. AFM is often less time-consuming and less expensive than TEM or SEM, and air samples or liquid dispersions can be observed by this technique. The sample must adhere to a substrate and the roughness of the substrate must be less than the size of the nanoparticles to be measured.

Microscopy methods are valuable for the characterization of nanostructures containing antimicrobial substances with potential food applications. The spherical

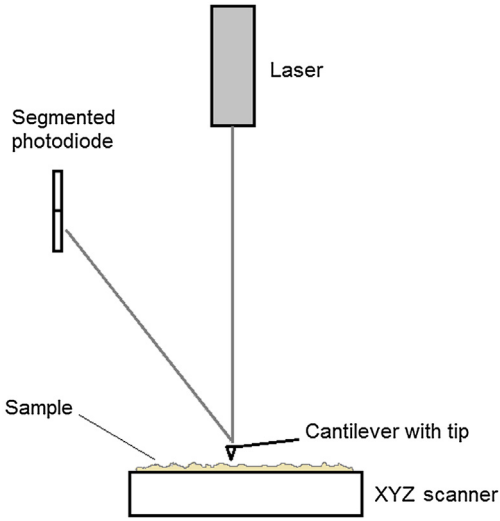

Figure 11.4 Schematic representation of an atomic force microscopy device.

morphology of nanoliposomes containing the antimicrobial peptides nisin and P34 and their interactions with bacteria were demonstrated by TEM studies (Colas et al., 2007; Malheiros, Sant'Anna, Micheletto, Silveira, & Brandelli, 2011). In addition, SEM is an essential technique for the structural characterization of composite films intended for food use, in which nanometric materials are frequently incorporated (Rodriguez, Sepulveda, Bruna, Guarda, & Galotto, 2012; Zehetmeyer, Soares, Brandelli, Mauler, & Oliveira, 2012).

The major criticism of the microscopy methods for nanoparticle characterization is the difficulty of sample preparation (Table 11.3). Some criticism has been also related to the resolution of crystalline samples and whether individual crystal particles are correctly identified. A small sample is chosen for viewing and this may not be an average of the sample as a whole. Thus, it is important to select a "representative" sample to study.

11.4.3 Zeta potential

The zeta potential reflects the surface potential (electrophoretic mobility) of particles, which is influenced by the interfacial changes with the medium as a function of dissociation of functional groups at the surface of the particles or adsorption of ionic species that are present in the dispersing medium (Hunter, 1988). This parameter is determined through electrophoretic techniques. Phospholipid (lecithins) and polymer constituents of nanoparticles are the main components of the formulations that are capable of influencing the zeta potential. In particular polyesters, such as PLA, and lecithins give a negative potential to the interface, whereas the poloxamers (nonionic tensoactives/surfactants) tend to reduce the absolute value of this parameter. In

Table 11.3 Advantages and disadvantages of microscopy methods for nanostructure characterization

Technique	Advantages	Drawbacks
Electron microscopy	Very high resolution (<1 nm) Direct method No calibration is needed Can be directly combined with analytical methods Any particle shape is accessible	Expensive and complex equipment Power source needs to be highly stable High vacuum is needed Difficult sample preparation Time consuming Poor statistics Artifacts
Atomic force microscopy	Very high resolution Fast Available equipment Inexpensive	Strong influence of the tip (size, shape, material) Influence of the nanoparticulate material (tip−particle interaction) Particles need to be on a surface Complex statistics

modulus, a relatively high zeta potential value is important to provide a good physicochemical stability of a colloidal suspension, because high repulsion forces are useful to avoid particle aggregation as a function of occasional collision of neighboring nanoparticles (Tantra, Schulze, & Quincey, 2010).

The surface characteristics of the particles may also alter the biological response of the antimicrobial associated with the nanoparticle. Various strategies have been proposed to improve the stability and distribution of nanoparticles, based mainly on the reduction of surface hydrophobicity of the particles through the physical adsorption of a hydrophilic polymer or alteration of surface charge. The phospholipid composition of liposomes influences the zeta potential. Nanoliposomes formed by charged polar lipids can be expected to be more stable than those composed of neutral polar lipids, because elevated zeta potential/surface charge density increases the repulsive interactions, reducing the frequency of liposome collisions. For instance, nanoliposomes composed by phosphatidylcholine showed a zeta potential of −8.3 mV, but these values reached −52.3 and −72.6 mV when phosphatidylglycerol was added to the formulation at 8:2 and 6:4 proportion, respectively (Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007).

11.4.4 Physicochemical methods

In addition to the nanostructure characterization, knowledge about the association of the antimicrobial with the nanomaterial constitutes relevant information. Some of the techniques that are useful to this aim are differential scanning calorimetry (DSC), XRD, FTIR, mass spectroscopy, and NMR.

Thermoanalytical methods, such as DSC, are very useful for the analysis of polymers and have been also used to investigate the interactions between polymers and drugs in several nanoparticle formulations. Useful information can be obtained regarding the morphology of crystalline polymers and about the dispersion state of the substance associated with this polymer. DSC analyses have been also used to study the intermolecular interactions among drugs and adjuvants, being useful to determine potential physical or chemical incompatibilities between them. It is also possible to investigate chemical reactions such as polymerization, depolymerization, and degradation (Wu, Zhang, & Watanabe, 2011).

The characterization of the physical state of a drug associated with nanostructures formed by synthetic polymers by DSC and XRD may indicate that the drug is dispersed molecularly in the polymeric matrix. In addition, the use of FTIR allows the examination of the absence of chemical reactions between the drug and the polymer (Wu et al., 2011). In addition to the usefulness of DSC, XRD, and FTIR in the elucidation of the association of the antimicrobial with the nanostructures, these techniques can also be applied to obtain information regarding the organization of other compounds in the formulation.

11.4.5 Characterization of antimicrobial activity

Despite the essential relevance of the determination of size, morphology, and physicochemical properties of nanostructures, the adequate evaluation of their biological activities is also a point of major concern. Regarding the nanostructures incorporating antimicrobial substances, or presenting antimicrobial activity by themselves, it is very important to have adequate methods to characterize the antimicrobial activity. Testing for antimicrobial activity can be either quantitative or qualitative. In quantitative tests, the minimum amount of the antimicrobial substance that inhibits the visible growth of a microbial isolate or minimal inhibitory concentration (MIC) is determined. Qualitative tests characterize a microbial isolate only as susceptible or not to a particular antimicrobial agent. Most of the conventional techniques used to evaluate antimicrobial susceptibility can be used or easily adapted to determine the antimicrobial activity of nanoparticles. In this regard, agar diffusion techniques, direct plate count, and determination of the MIC have been used to characterize the antimicrobial activity of silver nanoparticles (Dar, Ingle, & Raj, 2013), antibiotic-loaded nanoparticles (Rai, Prabhune, & Perry, 2010), and also nanostructures carrying natural antimicrobials (Malheiros et al., 2010).

Standard antimicrobial susceptibility testing by agar diffusion methods is often performed on Mueller–Hinton agar plates. In the disk diffusion test, the selected microorganism is spread over the surface of an agar plate, and then the antimicrobial is disposed onto a disk, which is placed on the agar surface. Then, the plates are incubated and the presence of inhibitory zones without microbial growth is examined and measured around the disks (Hindler, Howard, & Keiser, 1994). This technique is based on the original Bauer–Kirby test, and the diameter around each disk is interpreted as susceptible, intermediate, or resistant based on standards indicated by the Clinical Laboratory Standards Institute (CLSI, 2013). Alternatively, antimicrobial

activity can be determined by agar well diffusion test. In this method, the antimicrobial is directly loaded into wells of a defined diameter cut in the agar gel. This eliminates the need of impregnating disks with the antimicrobial to be tested, and the technique may be more sensitive than the disk diffusion method (Valgas, Souza, Smânia, & Smânia, 2007). Agar diffusion tests may offer a semiquantitative approach to the antimicrobial activity, but the diameter of the inhibition zone does not always correspond to a direct relationship with the antimicrobial concentration. Agar diffusion methods may have the limitations associated with impaired migration from disk to medium and diffusion through the agar matrix, which are dependent on the properties of the substance tested and the structure and diameter of the pores within the gelatinized medium.

The direct plate count is a method used to enumerate the number of viable cells in a sample. By the nature of the procedure, only live cells are included in the count. There are two main methods of direct plate counting: the spread-plate method and the pour-plate method (Madigan, Martinko, & Parker, 2000). The spread-plate method consists of uniformly spreading a volume no greater than 0.1 ml of the diluted sample over an agar plate. Volumes greater than 0.1 ml are not usually used to ensure that the liquid that is spread soaks in. The plate is then incubated until the colonies appear on the surface of the agar, and the number of colonies is counted. In the pour-plate method, a diluted sample is pipetted into a sterile petri plate, and then melted agar is poured in and mixed with the sample. Using this method allows for a larger volume of the diluted sample, usually in the range of 0.1−1.0 ml. This method yields colonies throughout the agar, and the organism to be counted must be able to briefly withstand the temperatures associated with the melted agar. Direct plate count methods have been used to characterize the antimicrobial activity of nano-antimicrobial materials as well. Most studies regarding the investigation of silver nanoparticles have used *E. coli* as the model test bacterium, but testing against other Gram-negative, Gram-positive, and also multidrug-resistant strains has also been described (Chapman, Sullivan, & Regan, 2012). In addition, direct plate count has been used for the evaluation of the antimicrobial activity of copper nanoparticles against diverse bacteria (Diaz-Visurraga et al., 2012), antimicrobial peptide-loaded PLGA nanoparticles against *C. neoformans* (Che, Wu, Xu, Xu, & Chen, 2009), and ampicillin-functionalized nanoparticles against multiresistant *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and methicillin-resistant *S. aureus* (Brown et al., 2012).

Standard methods described by the CLSI and ASTM E2315 are recommended for the quantitative measurement of the inhibitory activity of antimicrobial substances. Determination of MIC is used to examine at which concentrations an antimicrobial substance can inhibit a microbial strain. Variable concentrations, in general serial twofold dilutions, of the antimicrobial agent are added to broth, which are inoculated with a standardized suspension of the test microorganism. After overnight incubation, the MIC is the lowest concentration that inhibits growth as evidenced by the absence of visual turbidity (Hindler et al., 1994).

The minimal bactericidal concentration (MBC) is basically an extension of the MIC test and requires an additional step that involves a colony count on the actual inoculum. After the MIC test is performed, every tube that showed no growth (concentrations

equal to and greater than MIC) is subcultured onto an agar medium to determine if the initial inoculum was killed or merely inhibited. The end point is defined as the lowest concentration that kills 99.9% or more of the test microorganism (Hindler et al., 1994). Another method for assessing the bactericidal activity of an antimicrobial agent is the time−kill assay. This test is more laborious than the MBC test and is mainly performed in research settings when a novel antimicrobial is being evaluated, to confirm unusual MBC observations, or as an effort to explain treatment failure when bactericidal action is mandatory. In this test, a standardized number of bacteria in the midexponential growth phase are added to one or more concentrations of antimicrobial in broth. Tests are often performed in larger broth volumes (>10 ml) and the drug concentration is usually the MIC, twofold MIC, and fourfold MIC. Colony counts are performed on 0.1-ml aliquots removed from each tube at various times. The counts of viable cells remaining are plotted against time, and generally a three or more $\log_{10}$ reduction in bacterial counts in the suspension containing the antimicrobial agent in comparison with the control indicates an adequate bactericidal response.

Fluorescent probes that are used to evaluate injury of microbial cells can be also employed as helpful tools to assess the effects of nanoparticles. Increased fluorescence uptake of 1-*N*-phenylnaphthylamine occurs in bacterial suspensions containing cells whose outer membrane is damaged and functionally invalid. The membrane potential-sensitive cyanine dye $DiSC_3(5)$ fits into the membrane with high membrane potential gradient and then forms aggregates that involve self-quenching, resulting in decreased fluorescence. Membrane disruptions generate membrane potential dissipation and then $DiSC_3(5)$ is released into the medium, causing an increased fluorescence. The fluorescent redox probes 5-cyano-2,3-ditolyltetrazolium chloride and 4′,6-diamidino-2-phenylindole are used for enumerating actively respiring cells (Yaqub, Anderson, MacGregor, & Rowan, 2004). These fluorescent probes have been used to assess the effects of nanoparticles against bacterial cells as well (Pelletier et al., 2010; Xing et al., 2009).

11.5 Food applications of nanostructured antimicrobial systems

Despite several studies having been published regarding the production and characterization of stable nanoparticles incorporating antimicrobials, there are relatively few data about the application of such antimicrobial-loaded nanoparticles in foods, or even in model food systems.

Earlier studies on the encapsulation of the bacteriocin pediocin AcH within phosphatidylcholine liposomes resulted in an increase in the recovery of pediocin activity in slurries of heated beef muscle, beef tallow, nonfat dry milk, and butterfat compared with slurries containing free pediocin (Degnan, Buyong, & Luchansky, 1993). Interestingly, a greater improvement of pediocin activity was observed in dairy-based compared to meat-based slurries. Such results demonstrate the efficacy of encapsulation in enhancing antimicrobial activity, minimizing the negative effects observed for

Table 11.4 Incorporation of nisin into nanoliposomes

Nanoparticle composition	Mean particle size (nm)	Target microorganism
Soy lecithin	148–190	*L. monocytogenes*
Proliposomes (Pro-lipo®, Duo S and H), with or without Cho	140–2400	*L. monocytogenes*
DPPC, phospholipon 90H and 100H, with or without Cho DCP, SA	190–284	*B. subtilis*, *P. aeruginosa*
PC, PC:PG (8:2 and 6:4)	123–310	*L. monocytogenes*, *E. coli* O157:H7
PC, PC:Cho (7:3), PC:PG:Cho (5:2:3)	131–182	*L. monocytogenes*
PC, PC:Cho (7:3)	158–218	*Listeria* spp.
Proliposome H	80–120	*L. innocua*

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; DPPC, dipalmitoyl-PC; Cho, cholesterol; SA, stearylamine; DCP, dicetylphosphate.

nonencapsulated antimicrobials, such as proteolysis and association with protein and/or fat present in food.

Most reports on the incorporation of natural food antimicrobials in nanostructures could be related to nanovesicles containing nisin or lysozyme (Malheiros et al., 2010; Were, Bruce, Davidson, & Weiss, 2003, 2004). The incorporation of nisin into nanostructures has been reported by various authors (Table 11.4). Various nanoliposomes containing nisin are described, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and mixed phospholipids, also containing cholesterol in the formulation (Taylor et al., 2007; Were et al., 2003). The major goal behind the incorporation of this bacteriocin in nanostructures is to avoid undesirable interaction with some food components and to achieve controlled release, thus an increased shelf life could be expected. In this regard, encapsulation of nisin and ethylenediaminetetraacetic acid (EDTA) in phosphatidylcholine/phosphatidylglycerol nanoliposomes caused a significant increase in the lag phases of *L. monocytogenes* and *E. coli* O157:H7, resulting in bacteriostatic inhibition of these pathogens for at least 48 h (Taylor, Bruce, Weiss, & Davidson, 2008). The addition of encapsulated nisin to fluid milk resulted in a significant increase in the lag phase of *L. monocytogenes* Scott A incubated at 5 or 20 °C (Schmidt, Holub, Sturino, & Taylor, 2009). Similar bacteriostatic inhibition of *L. monocytogenes* was observed in whole or skimmed fluid milk with added nanoencapsulated nisin and maintained at 7 or 30 °C (Malheiros, Daroit, Silveira, & Brandelli, 2010). The preparation of SLNs also appears to be a good system for nisin encapsulation. The production of nisin-containing SLNs by high-pressure homogenization resulted in nanoparticles showing antibacterial activity against *L. monocytogenes* for up to 20 days (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012). Another GRAS bacteriocin, pediocin PA-1/AcH, has

also been incorporated into phosphatidylcholine nanovesicles, showing antimicrobial activity against diverse *Listeria* spp. (Mello et al., 2013).

Lysozyme has been used as a model protein for incorporation in nanostructures. Full lysozyme activity was preserved when it was used as a positively charged model protein to investigate the viability of negatively charged nanoparticles, consisting of polymer blends of PLGA and poly(styrene-co-4-styrene sulfonate) and to improve the loading capacity and release properties (Cai, Bakowsky, Rytting, Schaper, & Kissel, 2008). Colloidal suspensions of lysozyme and silver nanoparticles were used as antimicrobial coatings on medical instruments, effectively reducing the cell viability of *Klebsiella pneumoniae*, *Bacillus anthracis* Sterne, *S. aureus*, and *Acinetobacter baylyi* (Eby, Luckarift, & Johnson, 2009). Another study demonstrated that composite nanoparticles of lysozyme coated with poly-γ-glutamic acid and chitosan have outstanding antibacterial activity (Liu et al., 2013). The antimicrobial protein lactoferrin was also encapsulated into nanoparticles. Lactoferrin encapsulated in stable NEs was capable of inhibiting *S. aureus*, *Listeria innocua*, and *Candida albicans* (Balcão et al., 2013), whereas its encapsulation in nanoliposomes prepared by the reversed-phase method resulted in decreased viability of Caco-2 cells, suggesting a potential model to target lactoferrin to cancer cells (Guan et al., 2012).

The encapsulation of plant-derived natural antimicrobials has also been reported. Liposomes of phosphatidylcholine/cholesterol incorporating thymol and carvacrol resulted in enhanced antimicrobial activity against *L. monocytogenes*, *Staphylococcus* spp., *Pseudomonas* spp., and *Candida* spp. (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009). Also, PLGA nanoparticles incorporating eugenol and *trans*-cinnamaldehyde were produced by the emulsion evaporation method. These nanoparticles showed slow release of the antimicrobial compounds and efficient inhibition of *Listeria* spp. and *Salmonella* spp. (Gomes, Moreira, & Castell-Perez, 2011). A terpene mixture and D-limonene were encapsulated into NEs based on food-grade ingredients, prepared by high-pressure homogenization. When the nanocapsules were tested in pear and orange juices inoculated with *Lactobacillus delbrueckii*, low antimicrobial concentrations were required for a bactericidal action owing to the higher antimicrobial activity of the nanoencapsulated compounds (Donsi, Annunziata, Sessa, & Ferrari, 2011). The results of nanoencapsulation of natural antimicrobial substances in both natural and synthetic polymers indicate that this strategy can be used to control spoilage and pathogenic microorganisms and increase food stability.

11.6 Conclusions and future trends

Nanostructures are promising tools for the delivery and controlled release of natural antimicrobials in food. Several natural food antimicrobials such as nisin and lysozyme have been successfully incorporated into nanostructures. Other natural antimicrobials have been also tested, showing effective inhibition of a variable range of food-related microorganisms. However, most studies have been developed *in vitro* and, therefore, the technological aspects for incorporation of antimicrobial

nanostructures in food products must be a point of further investigation. Although few studies on food systems and real food samples are available, these indicate that nanostructures are promising tools for incorporation of natural antimicrobial compounds into food.

Despite many foods containing nanoscale components, which have been eaten safely for a long time, the toxicological aspects of most formulated nanostructures that could be added to food systems are yet poorly explored. There is a scientific consensus that nanomaterials are essentially different substances that create new and unique risks to human health and environment and require specific forms of safety monitoring. Thus, the understanding of food components at the nanometric scale is important to create innovative food products and technologies, with the guarantee of food safety and quality. Nanotechnology presents many valuable applications to food science and technology, and the correct evaluation and management of nanomaterials are essential to the inclusion of this technology by the food industry.

References

Almeida, A. J., & Souto, E. (2007). Solid lipid nanoparticles as drug delivery system for peptides and proteins. *Advanced Drug Delivery Reviews*, *59*, 478−490.

Alsan, S., Deneufchatel, M., Hashmi, S., Li, N., Pfefferle, L. D., Elimelech, M., et al. (2012). Carbon nanotube-based antimicrobial biomaterials formed via layer-by-layer assembly with polypeptides. *Journal of Colloid and Interface Science*, *388*, 268−273.

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection*, *1*, 125−129.

Baker, J. R., Hamouda, T., Shih, A., & Myc, A. (2000). *Methods of preventing and treating microbial infections*. The Regents of the University of Michigan. US Patent 6,506,803.

Balcão, V. M., Costa, C. I., Matos, C. M., Moutinho, C. G., Amorim, M., Pintado, M. E., et al. (2013). Nanoencapsulation of bovine lactoferrin for food and biopharmaceutical applications. *Food Hydrocolloids*, *32*, 425−431.

Brandelli, A. (2012). Nanostructures as promising tools for delivery of antimicrobial peptides. *Mini-Reviews in Medicinal Chemistry*, *12*, 731−741.

Brannon-Peppas, L., & Blanchette, J. O. (2012). Nanoparticle and targeted systems for cancer therapy. *Advanced Drug Delivery Reviews*, *64*, S206−S212.

Breukink, E., & de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta*, *1462*, 223−234.

Brown, A. N., Smith, K., Samuels, T. A., Lu, J., Obare, S. O., & Scott, M. E. (2012). Nanoparticles functionalized with ampicillin destroy multiple-antibiotic-resistant isolates of *Pseudomonas aeruginosa* and *Enterobacter aerogenes* and methicillin-resistant *Staphylococcus aureus*. *Applied and Environmental Microbiology*, *78*, 2768−2774.

Cai, C., Bakowsky, U., Rytting, E., Schaper, A. K., & Kissel, T. (2008). Charged nanoparticles as protein delivery systems: a feasibility study using lysozyme as model protein. *European Journal of Pharmaceutics and Biopharmaceutics*, *69*, 31−42.

Chapman, J., Sullivan, T., & Regan, F. (2012). Biological methods for characterization of nano-anti-microbial materials. In *Nanoparticles in anti-microbial materials: Use and characterization* (pp. 153−193). RSC Nanoscience and Nanotechnology Series no 23. The Royal Society of Chemistry.

Che, F. B., Wu, Y., Xu, N., Xu, N., & Chen, J. H. (2009). Killer peptide-loaded nanoparticles: characterization and evaluation of anticryptococcal efficacy. *Academic Journal of Second Military Medical University*, *30*, 505−508.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*, 1−20.

CLSI. (2013). *M100−S63 performance standards for antimicrobial susceptibility testing*. Wayne: Clinical Laboratory Standard Institute.

Colas, J. C., Shi, W., Rao, V. S., Omri, A., Mozafari, M. R., & Singh, H. (2007). Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. *Micron*, *38*, 841−847.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*, 564−582.

Dar, M. A., Ingle, A., & Raj, M. (2013). Enhanced antimicrobial activity of silver nanoparticles synthesized by *Cryphonectria* sp. evaluated singly and in combination with antibiotics. *Nanomedicine*, *9*, 105−110.

Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annual Review of Food Science Technology*, *4*, 163−190.

Degnan, A. J., Buyong, N., & Luchansky, J. B. (1993). Antilisterial activity of pediocin AcH in model food systems in the presence of an emulsifier or encapsulated within liposomes. *International Journal of Food Microbiology*, *18*, 127−138.

Díaz-Visurraga, J., Daza, C., Pozo, C., Becerra, A., von Plessing, C., & García, A. (2012). Study on antibacterial alginate-stabilized copper nanoparticles by FT-IR and 2D-IR correlation spectroscopy. *International Journal of Nanomedicine*, *7*, 3597−3612.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT Food Science and Technology*, *44*, 1908−1914.

Doores, S. (2005). Organic acids. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in foods* (pp. 91−142). New York: Marcel Dekker.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, *70*, 564−582.

Eby, D. M., Luckarift, H. R., & Johnson, G. R. (2009). Hybrid antimicrobial enzyme and silver nanoparticle coatings for medical instruments. *ACS Applied Material and Interfaces*, *1*, 1553−1560.

Egerton, R. F. (2005). *Physical principles of electron microscopy*. New York: Springer.

Eijsink, V. G. H., Skeie, M., Middelhoven, P. M., Bruberg, M. B., & Nes, I. F. (1998). Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Applied and Environmental Microbiology*, *64*, 3275−3281.

Emerich, D. F., & Thanos, C. G. (2006). The pinpoint promise of nanoparticle-based drug delivery and molecular diagnosis. *Biomolecular Engineering*, *23*, 171−184.

Gálvez, A., Abriouel, H., López, R. L., & Omar, N. B. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology*, *120*, 51−70.

Gibbs, P. A. (1987). Novel uses for lactic acid fermentation in food preservation. *Journal of Applied Microbiology*, *63*, S51−S58.

Gomes, C., Moreira, R. G., & Castell-Perez, E. (2011). Poly(DL-lactide-co-glycolide) (PLGA) nanoparticles with entrapped *trans*-cinnamaldehyde and eugenol for antimicrobial delivery applications. *Journal of Food Science*, *76*, S16−S24.

Grau, A., Gómez-Fernández, J. C., Peypoux, F., & Ortiz, A. (2001). Aggregational behaviour of aqueous dispersions of the antifungal lipopeptide iturin A. *Peptides*, *22*, 1−5.

Guan, R., Ma, J., Wu, Y., Lu, F., Xiao, C., Jiang, H., et al. (2012). Development and characterization of lactoferrin nanoliposome: cellular uptake and stability. *Nanoscale Research Letters*, *7*, 679.

Guterres, S. S., Alves, M. P., & Pohlmann, A. R. (2007). Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. *Drug Target Insights*, *2*, 147−157.

Hamouda, T., Hayes, M. M., Cao, Z., Tonda, R., Johnson, K., Wright, C. D., et al. (1999). A novel surfactant nanoemulsion with broad-spectrum sporicidal activity against *Bacillus* species. *Journal of Infectious Disease*, *180*, 1939−1949.

Heunis, T. D. J., Botes, M., & Dicks, L. M. T. (2010). Encapsulation of *Lactobacillus plantarum* 423 and its bacteriocin in nanofibers. *Probiotics and Antimicrobial Proteins*, *2*, 46−51.

Heunis, T. D. J., & Dicks, L. M. T. (2010). Nanofibers offer alternative ways to the treatment of skin infections. *Journal of Biomedicine and Biotechnology*, *2010*, 510682.

Hindler, J. A., Howard, B. J., & Keiser, J. F. (1994). Antimicrobial agents and antimicrobial susceptibility testing. In B. J. Howard (Ed.), *Clinical and pathogenic microbiology* (pp. 145−210). St. Louis: Mosby-Year Book.

Hunter, R. J. (1988). *Zeta potential in colloid science: Principles and applications*. London: Academic Press.

Jesorka, A., & Orwar, O. (2008). Liposomes: technologies and analytical applications. *Annual Review of Analytical Chemistry*, *1*, 801−832.

Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel natural food antimicrobials. *Annual Review of Food Science and Technology*, *3*, 381−403.

Kim, T. G., & Park, T. G. (2006). Surface functionalized electrospun biodegradable nanofibers for immobilization of bioactive molecules. *Biotechnology Progress*, *22*, 1108−1113.

Lam, C. W., James, J. T., McCluskey, R., Arepalli, S., & Hunter, R. L. (2006). A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Critical Reviews in Toxicology*, *36*, 189−217.

Legrand, D., & Mazurier, J. (2010). A critical review of the roles of host lactoferrin in immunity. *Biometals*, *23*, 365−376.

Liolios, C. C., Gortzi, O., Lalas, S., Tsaknis, J., & Chinou, I. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and *in vitro* antimicrobial activity. *Food Chemistry*, *112*, 77−83.

Liu, L. H., Xu, K. J., Wang, H. Y., Tan, P. K. J., Fan, W. M., Venkatraman, S. S., et al. (2009). Self-assembled cationic peptide nanoparticles as an efficient antimicrobial agent. *Nature Nanotechnology*, *4*, 457−463.

Liu, Y., Sun, Y., Xu, Y. X., Feng, H., Fu, S. D., Tang, J. W., et al. (2013). Preparation and evaluation of lysozyme-loaded nanoparticles coated with poly-γ-glutamic acid and chitosan. *International Journal of Biological Macromolecules*, *59*, 201−207.

Lönnerdal, B., & Iyer, S. (1995). Lactoferrin: molecular structure and biological function. *Annual Review of Nutrition*, *15*, 93−110.

Lovelyn, C., & Attama, A. A. (2011). Current state of nanoemulsions in drug delivery. *Journal of Biomaterials and Nanobiotechnology*, *2*, 626−639.

Madigan, M. T., Martinko, J. M., & Parker, J. (2000). *Biology of microorganisms* (9th ed.). Upper Saddle River, NJ: Prentice Hall.

Malheiros, P. S., Daroit, D. J., & Brandelli, A. (2010). Food applications of liposome-encapsulated antimicrobial peptides. *Trends in Food Science and Technology*, *21*, 284−292.

Malheiros, P. S., Daroit, D. J., Silveira, N. P., & Brandelli, A. (2010). Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk. *Food Microbiology*, *27*, 175−178.

Malheiros, P. S., Micheletto, Y. M. S., Silveira, N. P., & Brandelli, A. (2010). Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International*, *43*, 1198−1203.

Malheiros, P. S., Sant'Anna, V., Micheletto, Y. M. S., Silveira, N. P., & Brandelli, A. (2011). Nanovesicle encapsulation of antimicrobial peptide P34: physicochemical characterization and mode of action on *Listeria monocytogenes*. *Journal of Nanoparticle Research*, *13*, 3545−3552.

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., & Graves, S. M. (2006). Nanoemulsions: formulations, structure, and physical properties. *Journal of Physics: Condensed Matter*, *18*, R635−R666.

Masschalck, B., & Michiels, C. W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Critical Reviews in Microbiology*, *29*, 191−214.

Maretschek, S., Greiner, A., & Kissel, T. (2008). Electrospun biodegradable nanofiber nonwovens for controlled release of proteins. *Journal of Controlled Release*, *127*, 180−187.

McAuliffe, O., Ross, R. P., & Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*, 285−308.

Mello, M. B., Malheiros, P. S., Brandelli, A., Silveira, N. P., Jantzen, M. M., & Motta, A. S. (2013). Characterization and antilisterial effect of phosphatidylcholine nanovesicles containing the antimicrobial peptide pediocin. *Probiotics and Antimicrobial Proteins*, *5*, 43−50.

Mozafari, M. R., Johnson, C., Hatziantoniou, S., & Demetzos, C. (2008). Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research*, *18*, 309−327.

Muriana, P. M., & Klaenhammer, T. R. (1991). Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Applied and Environmental Microbiology*, *57*, 114−121.

Nagao, J., Asaduzzaman, S. M., Aso, Y., Okuda, K., Nakayama, J., & Sonomoto, K. (2006). Lantibiotics: insight and foresight for new paradigm. *Journal of Bioscience and Bioengineering*, *102*, 139−149.

Naidu, A. S., & Davidson, P. M. (2000). Phytophenols. In A. S. Naidu (Ed.), *Natural food antimicrobial systems* (pp. 265−294). Boca Raton, FL: CRC Press.

Nepal, D., Balasubramanian, S., Simonian, A. L., & Davis, V. A. (2008). Strong antimicrobial coatings: single-walled carbon nanotubes armored with biopolymers. *Nano Letters*, *8*, 1896−1901.

Pangule, R. C., Brooks, S. J., Dinut, C. Z., Bale, S. S., Salmon, S. L., Zhu, G., et al. (2010). Antistaphylococcal nanocomposite films based on enzyme-nanotube conjugates. *ACS Nano*, *4*, 3993−4000.

Pelletier, D. A., Suresh, A. K., Holton, G. A., McKeown, C. K., Wang, W., Gu, B., et al. (2010). Effects of engineered cerium oxide nanoparticles on bacterial growth and viability. *Applied and Environmental Microbiology*, *76*, 7981−7989.

Piper, C., Hill, C., Cotter, P. D., & Ross, R. P. (2011). Bioengineering of nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants nisin F, Q and Z. *Microbial Biotechnology*, *4*, 375−382.

Povey, M. J. W. (2013). Ultrasound particle sizing: a review. *Particuology*, *11*, 135−147.

Prombutara, P., Kulwatthanasal, Y., Supaka, N., Sramala, I., & Chareonpornwattana, S. (2012). Production of nisin-loaded solid lipid nanoparticles for sustained antimicrobial activity. *Food Control*, *24*, 184−190.

Pusey, P. N. (1999). Suppression of multiple scattering by photon cross-correlation techniques. *Current Opinion in Colloid and Interface Science*, *4*, 177−185.

Rai, A., Prabhune, A., & Perry, C. C. (2010). Antibiotic mediated synthesis of gold nanoparticles with potent antimicrobial activity and their application in antimicrobial coatings. *Journal of Material Chemistry*, *20*, 6789−6798.

Reches, M., & Gazit, E. (2006). Molecular self-assembly of peptide nanostructures: mechanism of association and potential uses. *Current Nanoscience*, *2*, 105−111.

Rodriguez, F., Sepulveda, H. M., Bruna, J., Guarda, A., & Galotto, M. J. (2012). Development of cellulose eco-nanocomposites with antimicrobial properties oriented for food packaging. *Packaging Technology and Science*, *26*, 149−160.

Ruf, H. (1993). Data accuracy and resolution in particle sizing by dynamic light scattering. *Advances in Colloid and Interface Science*, *46*, 333−342.

Saifuddin, N., Raziah, A. Z., & Junizah, A. R. (2013). Carbon nanotubes: a review on structure and their interaction with proteins. *Journal of Chemistry*, *2013*, 676815.

Santoso, S. S., Vauthey, S., & Zhang, S. (2002). Structures, function and applications of amphiphilic peptides. *Current Opinion in Colloid and Interface Science*, *7*, 262−266.

Schmidt, S. E., Holub, G., Sturino, J. M., & Taylor, T. M. (2009). Suppression of *Listeria monocytogenes* Scott A in fluid milk by free and liposome-entrapped nisin. *Probiotics and Antimicrobial Proteins*, *1*, 152−158.

Shah, P., Bhalodia, D., & Shelat, P. (2010). Nanoemulsions: a pharmaceutical review. *Systemic Reviews in Pharmacy*, *1*, 24−32.

Shofran, B. G., Purrington, S. T., Breidt, F., & Fleming, H. P. (1998). Antimicrobial properties of sinigrin and its hydrolysis products. *Journal of Food Science*, *63*, 621−624.

Sinha, M., Kayshik, S., Kaur, P., Sharma, S., & Singh, T. P. (2013). Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein. *International Journal of Peptides*, *2013*, 1−12.

Sun, X. Y., Shankar, R., Borner, H. G., Ghosh, T. K., & Spontak, R. J. (2007). Field-driven biofunctionalization of polymer fiber surfaces during electrospinning. *Advanced Materials*, *19*, 87−91.

Tantra, R., Schulze, P., & Quincey, P. (2010). Effect of nanoparticle concentration on zeta-potential measurement results and reproducibility. *Particuology*, *8*, 279−285.

Taylor, T. M., Bruce, B. D., Weiss, J., & Davidson, P. M. (2008). *Listeria monocytogenes* and *Escherichia coli* O157:H7 inhibition *in vitro* by liposome-encapsulated nisin and ethylene diaminetetraacetic acid. *Journal of Food Safety*, *28*, 183−197.

Taylor, T. M., Davidson, P. M., Bruce, B. D., & Weiss, J. (2005). Ultrasonic spectroscopy and differential scanning calorimetry of liposomal-encapsulated nisin. *Journal of Agricultural and Food Chemistry*, *53*, 8722−8728.

Taylor, T. M., Gaysinsky, S., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Characterization of antimicrobial-bearing liposomes by ζ-potential, vesicle size, and encapsulation efficiency. *Food Biophysics*, *2*, 1−9.

Teo, W. E., & Ramakrishna, S. (2006). A review on electrospinning design and nanofibre assemblies. *Nanotechnology*, *17*, R89−R106.

Valdés-Stauber, N., & Scherer, S. (1994). Isolation and characterization of linocin M18, a bacteriocin produced by *Brevibacterium linens*. *Applied and Environmental Microbiology*, *60*, 3809−3814.

Valgas, C., Souza, S. M., Smânia, E. F. A., & Smânia, A., Jr. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, *38*, 369−380.

Vauthier, C., & Bouchemal, K. (2009). Methods for the preparation and manufacture of polymeric nanoparticles. *Pharmaceutical Research*, *26*, 1025−1058.

Walker, J. G. (2012). Improved nano-particle tracking analysis. *Measurement Science Technology*, *23*, 065605.

Wang, H., Xu, K. J., Liu, L., Tan, J. P. K., Chen, Y., Li, Y., et al. (2010). The efficacy of self-assembled cationic antimicrobial peptide nanoparticles against *Cryptococcus neoformans* for the treatment of meningitis. *Biomaterials*, *31*, 2874−2881.

Wei, Q. F., Gao, W. D., Hou, D. Y., & Wang, X. Q. (2005). Surface modification of polymer nanofibres by plasma treatment. *Applied Surface Science*, *245*, 16−20.

Were, L. M., Bruce, B. D., Davidson, P. M., & Weiss, J. (2003). Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *Journal of Agricultural and Food Chemistry*, *51*, 8073−8079.

Were, L. M., Bruce, B. D., Davidson, P. M., & Weiss, J. (2004). Encapsulation of nisin and lysozyme in liposomes enhances efficacy against *Listeria monocytogenes*. *Journal of Food Protection*, *67*, 922−927.

Wu, L., Zhang, J., & Watanabe, W. (2011). Physical and chemical stability of drug nanoparticles. *Advanced Drug Delivery Reviews*, *63*, 456−469.

Xing, K., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S., & Park, H. J. (2009). Effect of oleoyl-chitosan nanoparticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of *Escherichia coli* and *Staphylococcus aureus*. *Carbohydrate Polymers*, *76*, 17−22.

Yadav, S. C., Kumari, A., & Yadav, R. (2011). Development of peptide and protein nanotherapeutics by nanoencapsulation and nanobioconjugation. *Peptides*, *32*, 173−187.

Yao, N., & Wang, Z. L. (2005). *Handbook of microscopy for nanotechnology*. New York: Kluwer Academic Publishers.

Yaqub, S., Anderson, J. G., MacGregor, S. J., & Rowan, N. J. (2004). Use of a fluorescent viability stain to assess lethal and sublethal injury in food-borne bacteria exposed to high-intensity pulsed electric fields. *Letters in Applied Microbiology*, *39*, 246−251.

Yoo, H. S., Kim, T. G., & Park, T. G. (2009). Surface-functionalized electrospun nanofibers for tissue engineering and drug delivery. *Advanced Drug Delivery Reviews*, *61*, 1033−1042.

Zehetmeyer, G., Soares, R. M. D., Brandelli, A., Mauler, R. S., & Oliveira, R. V. B. (2012). Evaluation of polypropylene/montmorillonite nanocomposites as food packaging material. *Polymer Bulletin*, *68*, 2199−2217.

Zhang, L., Pornpattananangkul, D., Hu, C.-M. J., & Huang, C.-M. (2010). Development of nanoparticles for antimicrobial drug delivery. *Current Medicinal Chemistry*, *17*, 585−594.

Zhang, S. G. (2003). Fabrication of novel biomaterials through molecular self-assembly. *Nature Biotechnology*, *21*, 1171−1178.

Modelling the effects of natural antimicrobials as food preservatives

12

A.K. Jaiswal, S. Jaiswal
Dublin Institute of Technology, Dublin, Ireland

12.1 Introduction

Food products are perishable by nature and require protection from spoilage during their preparation, storage, and distribution to give them desired shelf life. Spoilage or other changes lead to loss of shelf life, which may occur in any of the stages of acquisition of raw materials through to the eventual consumption of the finished product. The principal reactions that lead to spoilage, and that are therefore also the principal targets for effective preservation and control, include physical, chemical, enzymatic, and microbiological factors. Because food products are now often sold in areas of the world far remote from their production sites, the need for extended safe shelf life for these products has also expanded. Improvements in the cold distribution chain have made international trade of perishable foods possible, but refrigeration alone cannot ensure the quality and safety of all perishable foods (Holley & Patel, 2005).

The safety and quality of foodstuffs are paramount for consumer health and satisfaction. Preservatives are required to maintain quality, extend shelf life, and ensure safety of food products. Regulations by the U.S. Food and Drug Administration (FDA) have required processors to achieve a 5.0 $\log_{10}$ reduction in the numbers of the most resistant pathogens in their finished products. Therefore, most preservation techniques aim to control all forms of spoilage. Synthetic preservatives have an essential role in food preservation. Chemicals such as butylated hydroxytoluene, butylated hydroxyanisole, sodium benzoate, sodium nitrite, and potassium sorbate have been commonly used in food products. Research has confirmed that synthetic preservatives are associated with some side effects (Botterweck, Verhagen, Goldbohm, Kleinjans, & van den Brandt, 2000); therefore, legislation has restricted their use in various foods (Brul & Coote, 1999). Furthermore, consumer preferences are moving towards foods that contain lower levels of chemical preservatives, exhibit characteristics of fresh or natural products, and are microbiologically safe.

Application of heat is the most common method for food preservation, because of its ability to inactivate microorganisms and spoilage enzymes. However, no matter how minimal the heating source is, thermal processing can promote reactions that could affect overall quality of foods. Today, the demand for processed foods goes beyond the fundamental requirements of safety and shelf-stability. More emphasis is

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being placed on high-quality and value-added foods. Nonthermal methods such as addition of natural antimicrobial agents in food, high-pressure processing, ultrasound, ozone, pulsed electric field, and ultraviolet are increasingly gaining attention for food preservation. However, the use of naturally occurring antimicrobial agents to inhibit pathogen growth and prevent food spoilage has received special attention (Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2010). These compounds are naturally produced and isolated from various sources, including plants, animals, and microorganisms, in which they constitute part of their host defence system (Juneja, Dwivedi, & Yan, 2012). Many naturally occurring compounds, such as polyphenols, glucosinolates, nisin, and plant essential oils, have been widely studied and are reported to be effective in their potential role as antimicrobial agents against spoilage and pathogenic microorganisms (Cleveland, Montville, Nes, & Chikindas, 2001; Daglia, 2012; Jaiswal, Rajauria, Abu-Ghannam, & Gupta, 2011; Solórzano-Santos & Miranda-Novales, 2012).

12.2 Antimicrobial susceptibility assessment

The antibacterial susceptibility test is used to determine the efficacy of potential natural antimicrobials against various bacterial species. However, one should keep in mind that antimicrobial susceptibility tests should be easy to do, reproducible (i.e., the ability to yield the same result on repeat testing), sensitive, and specific (Lambert & Pearson, 2000). The usefulness of an antibacterial agent in combating food spoilage can be assessed by determining the noninhibitory concentration (NIC), the concentration above which the inhibitor begins to have a negative effect on growth, and the minimum inhibitory concentration (MIC), i.e., the minimum dose of the antibacterial agent required to inhibit the growth of the test bacterium as compared to control (Jaiswal, Varma, O'Neill, Duffy, & McHale, 2013; Lambert & Pearson, 2000; Tiwari et al., 2009). However, the MIC and NIC values of an antibacterial agent are dependent on experimental conditions, which include the incubation temperature, type of organism, and inoculum size (Lambert & Lambert, 2003; Lambert & Pearson, 2000). The standard antibacterial susceptibility test can be conveniently divided into qualitative (diffusion) and quantitative (dilution) methods. Common diffusion tests include agar well diffusion, in which target organisms embedded in an agar plate are exposed to an antibacterial agent using impregnated sterile disks of filter paper or by placing the antibacterial agent directly on the surface or into preformed wells (Álvarez-Fernández, Cancelo, Díaz-Vega, Capita, & Alonso-Calleja, 2013; Kim, Cho, & Han, 2013; Vásconez, Flores, Campos, Alvarado, & Gerschenson, 2009). After an appropriate period of exposure (18−24 h), the size of the zone of growth inhibition around the antibacterial delivery area is measured and considered the indicator of antibacterial effectiveness. However, the agar diffusion assay is costly and time-consuming, and the measurements tend to be more qualitative than quantitative. Dilution methods include agar dilution and broth micro/macrodilution, which reduce time and cost and increase sensitivity for detection of antibacterial activity. However, most of these evaluations are based on only one end point and do not reflect the time−killing process.

12.3 Mathematical modelling in food preservation

A predictive food microbiological model is a mathematical expression that describes the growth, survival, inactivation, or biochemical process of the food-borne organisms (Gonzales-Barron, 2011). It is based on the fact that the responses of microorganisms to environmental factors are reproducible and that, by characterizing environments in terms of these factors, it is possible from past observation to forecast the responses of microorganisms in other similar environments. Hence, they can be useful to improve microbial food safety and quality and are leading to the development of a quantitative understanding of microbial ecology of foods. While mathematical models are very useful decision analysis tools, it must be remembered that models are, at best, only a simplified representation of reality (Fakruddin, Mazumdar, & Mannan, 2011).

Traditional microbial enumeration techniques are time-consuming and therefore, mathematical microbial models are used to assess the potential for growth of microorganisms in foods during preservation (Bovill et al., 2000) and can be very useful to reduce the number of expensive and time-consuming experiments. Furthermore, time–kill curve studies better describe the dynamic behaviour of antibacterial activity and a more accurate MIC value can be obtained from them (Andraud, Chauvin, Sanders, & Laurentie, 2011; Mueller, de la Peña, & Derendorf, 2004). These curves are analysed using various mathematical models, usually based on the assumption that the relationship between antibacterial activity and antibacterial concentration has a sigmoid shape. Although most of the modelling studies are primarily focused on the effects of temperature on growth and survival of bacteria, or other factors that have an impact on bacterial growth, such as pH, water activity, etc., data on the use of natural antibacterial agents for inhibiting microbial growth and modelling the resulting kinetics are not very common, and most of the studies are based on an end-point MIC determination (Diao, Hu, Feng, Li, & Xu, 2013; Jordán, Lax, Rota, Lorán, & Sotomayor, 2013). This chapter focuses on the basic concept of mathematical modelling, which can be applied to estimate the effects of natural antimicrobials as food preservatives.

12.4 Types of models

In the last decades of the twentieth century, several mathematical models were developed to estimate the growth or inactivation of microorganisms in food, and subsequently several classifications were proposed. Models can be classified by the microbiological event into kinetic and probability models (Roberts, 1989), in empirical and mechanistic ways, or by the variables considered into primary, secondary, and tertiary models (Whiting & Buchanan, 1993).

12.4.1 Kinetic and probability models

Kinetic model explains the time taken for a specified growth/death response, in terms of environmental variables such as temperature, pH (Boekel, 1996) or relative

humidity, nutrient content, and antimicrobial properties. Kinetic models are useful in that they can be used to predict changes in microbial counts with time. Typical examples of a kinetic model include the Gompertz and square root models, which describe the rates of response such as specific growth rate, lag time, and maximum population density (McMeekin, Olley, & Ross, 1993; Whiting & Buchanan, 1994), or inactivation/survival models that describe destruction or survival over time (Xiong, Xie, Edmondson, & Sheard, 1999). They also include polynomial models based on surface response methodology by which experiments generally involve simultaneous estimation of the effects of several factors on microbial growth or death.

Probability models take advantage of the possibility that a particular event will occur in specific environments. For example, it can be used to model spore-forming bacteria, such as the probability of *Clostridium botulinum* survival in canned corned beef (Buchanan, 1993). The basis for probability modelling is the relationship between the growth of microbial cells and the physicochemical properties of the environment (Ross & McMeekin, 1995). Probability models specify only the probability of growth or toxin production and do not show the rate at which they occur (Roberts, 1989).

12.4.2 Empirical and mechanistic models

Another classification of models is mechanistic (i.e., explanatory, 'white box', or deductive) or empirical (i.e., descriptive, observational, 'black box', or inductive).

Empirical models are data driven and describe the interpretation without attempting to relate it to an underlying theory. Empirical models are concerned with practical consequence and simply describe data under experimental conditions in the form of a convenient mathematical relationship (Gibson & Hocking, 1997). Polynomial equations are common empirical models. Empirical sigmoidal-type models such as the modified Gompertz and logistic models have been used for fitting bacterial growth (Gupta, Cox, Rajauria, Jaiswal, & Abu-Ghannam, 2012).

Mechanistic models are built up from theoretical bases and allow interpretation of the response in terms of known phenomena and processes (Krist, Ross, & McMeekin, 1998). Mechanistic models are considered to be more preferable than the empirical ones, as they usually contain fewer parameters, fit the data better, and extrapolate more sensibly (Draper, 1988). Mechanistically derived models are easier to develop further as the quantity and quality of information from the analysed system increase, and mechanistic models are inherently superior to empirical models (Van Impe, Nicolaï, Martens, De Baerdemaeker, & Vandewalle, 1992; Van Impe, Nicolaï, Schellekens, Martens, & De Baerdemaeker, 1995; Zwietering, Rombouts, & Van'T Riet, 1993). The semimechanistic model of Baranyi-Roberts has been used for fitting bacterial growth (Gupta et al., 2012).

12.4.3 Primary, secondary, and tertiary models

Whiting and Buchanan (1993) proposed a three-level classification method described as primary, secondary, and tertiary mathematical models. Among them, the concept of the primary model is fundamental for predictive food microbiology.

Primary mathematical models estimate the response of the microorganism with time to a single set of conditions. The response can be either direct or indirect measures of microbial population density or products of microbial metabolism. Basically, the model is aimed at describing the kinetics of the process with as few parameters as possible while being able to accurately define different stages of growth. When the increase in population is plotted against time, the resulting curve usually has four stages: early stationary phase or lag phase, acceleration phase or log phase, stationary phase, and decline or death phase.

The objective of the primary model is to test the ability of a model to fit individual growth curves and estimate its various parameters. This then can generate information about the microorganisms under investigation, such as lag-phase duration (λ), generation time (GT), maximum population density (N_{max}), and exponential growth rate (μ_{max}) (Whiting, 1995; Whiting & Buchanan, 1993) (Figure 12.1).

Generally, growth curves are figured on a $\log_{10}$-based cell density as a function of time, as microbial growth is an exponential phenomenon, but sometimes a natural logarithm base is preferred. The exponential growth rate is defined as the steepest tangent to the exponential phase, so the tangent is at the inflexion point, whereas the lag phase can be explained as the time at which that extrapolated tangent line crosses the inoculum level (McMeekin et al., 1993). The rate of exponential growth of a bacterial culture is expressed as generation time, and also the doubling time of the bacterial population, and can be calculated from μ_{max} (Eqn (12.1)):

$$\text{Generation time}\left(\text{GT}\right) = \frac{\log_{10}2}{\mu_{max}} \tag{12.1}$$

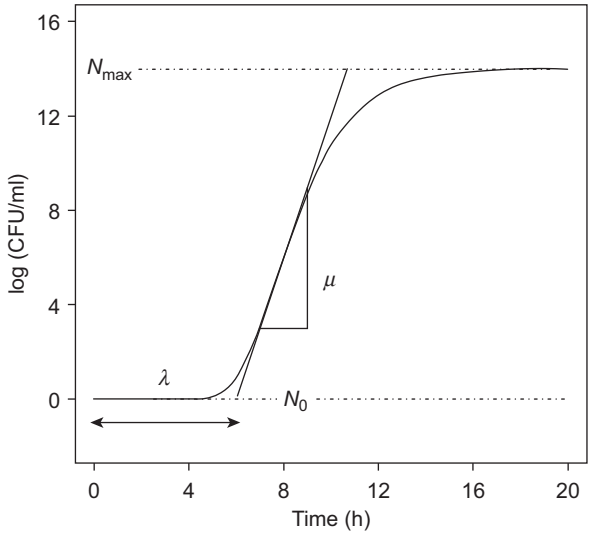

Figure 12.1 A typical growth curve and resulting parameters from the growth curves: lag-phase duration (λ), maximum population density (N_{max}), and exponential growth rate (μ_{max}). The integral (area under the curve) is also used as a growth parameter.
Source: Adapted from Isabelle and André (2006) with permission.

The first growth model was described by Monod and was based on a pure empirical observation that bacteria grow exponentially.

$$N = N_0 e^{kt} \tag{12.2}$$

where N is the microbial density (CFU/ml), N_0 is the initial microbial population (CFU/ml) and K is ln 2/GT (h^{-1}) and t is time (h). However, this model is limited as only the growth rate was considered for modelling, and lag phase was not modelled. Furthermore, important parameters such as maximum cell density were not taken into account.

Later, Gibson, Bratchell, and Roberts (1987) introduced the nonlinear Gompertz model, which makes it possible to express log (CFU/ml) as a function of time using a sigmoidal shape (Eqn (12.3)).

$$\log N_t = A + C \times \exp[-\exp[-\mathrm{B}(t-M)]] \tag{12.3}$$

where N_t is the cell density at time t; A is the lower asymptotic line of the growth curve as t decreases to zero, N_0, the initial population level at time $t = 0$ (log CFU/ml); C is the difference between the upper asymptotic line of the growth curve (maximum population level, N_{max}) and the lower asymptotic line (e.g., $N_{max} - N_0$) (log CFU/ml); B is the relative maximum growth rate (h^{-1}) at time M; and M is the time at which the growth rate is maximum. μ_{max} (h^{-1}) (Eqn (12.4)) and lag-phase duration (λ, h) (Eqn (12.5)) can be calculated by the equations

$$\mu_{max} = \frac{B \times C}{e} \tag{12.4}$$

where $e = 2.7182$, and

$$\lambda = M - \frac{1}{B} \tag{12.5}$$

$$N_{max} = A + C \tag{12.6}$$

In accordance with the Gompertz model, a logistic model (Eqn (12.7)) was proposed by Gibson et al. (1987) to predict the microbial growth.

$$N_t = A + \frac{C}{1 + \exp[-B \times (t - M)]} \tag{12.7}$$

where N_t, A, B, M, and C have the same meanings as specified for the Gompertz equation. The μ_{max} (Eqn (12.8)) and λ parameters (Eqn (12.9)) can be calculated as follows:

$$\mu_{max} = \frac{B \times C}{4} \tag{12.8}$$

$$\lambda = M - \frac{2}{B} \tag{12.9}$$

Model-fitting results are similar in both the cases; however, the Gompertz model was always preferred over the logistic model, as the former is symmetrical, whereas most growth curves are not.

In the 1990s, the focal point moved from the static primary model to dynamic primary models. Van Impe et al. (1992) proposed a first-order differential equation to predict microbial growth and inactivation. A second dynamic growth model was proposed by Baranyi and Roberts (1994). The model was based on first-order ordinary differential equation and is one of the most frequently used differential equation models to predict microbial growth. It can be described as in (Eqn (12.10)):

$$y(t) = y_0 + \mu_{\max} \mathrm{F(t)} - \ln\left(1 + \frac{e^{\mu_{\max}\mathrm{F(t)}} - 1}{e^{y_{\max}} - y_0}\right) \tag{12.10}$$

$$\text{where, } \mathrm{F(t)} = t + \frac{1}{v}\ln\left(e^{-vt} + e^{-h_0} - e^{-vt-h_0}\right) \tag{12.11}$$

where $y(t)$ is the $\log_e$ (CFU/ml) of cell concentration at time, t; y_0 is the initial cell concentration in $\log_e$ (CFU/ml) units; $y_{\max}$ is the maximum cell concentration in $\log_e$ (CFU/ml) units; $\mu_{\max}$ is the maximum specific growth rate in terms of $\log_e$ (CFU/ml), which is equal to $r_{\max}$ $\log_e$ 10 in 1 h^{-1}; v is the rate of increase of the limiting substrate, assumed to be equal to $\mu_{\max}$; h_0 is equal to $\mu_{\max} \times \lambda$; and λ is the lag-phase duration in h (Baranyi, Roberts, & McClure, 1993). Table 12.1 summarizes the most commonly used primary models that estimate the response of microorganisms.

Secondary mathematical models are produced from the primary model parameters such as lag time, growth/inactivation rate, and maximum population density. They indicate how parameters of the primary models change with respect to one or more environmental or cultural factors such as atmosphere, pH, temperature; antimicrobial agent concentration applied, and salt level. For example, if the effects of an antimicrobial agent on the growth of microorganisms were being investigated, the organism would be grown at a number of antimicrobial concentrations for specific periods such as 24−72 h. From each antimicrobial concentration, a generation time can be calculated by using a primary model. These data are then collated using a secondary model, so that the effects of antimicrobial concentrations are described by a mathematical equation. This allows the end user to determine what generation time will be observed at the respective antimicrobial concentrations.

Various secondary models have been applied to model growth and inactivation of microorganisms. These models can be simple linear regressions or more complex polynomial models, such as those based on response surface methodology that require sophisticated computational software (Khuri, 2011), square root models (Ratkowsky, Ross, McMeekin, & Olley, 1991), and Arrhenius equations. The Z value is another type of secondary model that describes the change in D value as a function of temperature. Generally, lag time and growth rate have been modelled

Table 12.1 The primary, secondary, and tertiary models most commonly used in the literature

Primary models	Secondary models	Tertiary models
Gompertz model[a]	Williams−Landel Ferry model[m]	USDA Pathogen Modeling Program[v]
Modified Gompertz[b]	Belehradek model (*square root model*)[n]	Microfit software[w]
Logistic model[c]	Ratkowsky model (*square root model*)[o]	Seafood Spoilage and Safety Predictor software[x]
Baranyi model[d]	Arrhenius model[p]	ComBase[y]
First-order monod model[e]	Modified Arrhenius models (*Davey or Schoolfield*)[q]	
Modified monod model[f]	Probability models[r]	
D Values of thermal inactivation[g]	Artificial neural Networks[s]	
Growth decline model of Whiting and Cygnarowicz[h]	*Z* values[t]	
Three-phase linear model[i]	Polynomial or response surface models[u]	
Richards model[j]		
Schnute model[k]		
Stannard model[l]		

[a]Gibson et al. (1987).
[b]Zwietering, Jongenburger, Rombouts, and Van't Riet (1990).
[c]Jason (1983).
[d]Baranyi et al. (1993).
[e]Monod (1949).
[f]Houtsma, Kant-Muermans, Rombouts, and Zwietering (1996).
[g]Juneja, Eblen, and Ransom (2001).
[h]Whiting and Cygnarowicz-Provost (1992).
[i]Buchanan, Whiting, and Damert (1997).
[j]Richards (1959).
[k]Schnute (1981).
[l]Stannard, Williams, and Gibbs (1985).
[m]Williams, Landel, and Ferry (1955).
[n]Běhrádek (1930).
[o]Ratkowsky, Olley, McMeekin, and Ball (1982).
[p]Arrhenius (1889).
[q]Davey (1993).
[r]Hauschild (1982).
[s]Marini (2009).
[t]Juneja, Snyder, and Marmer (1997).
[u]Khuri (2011).
[v]Juneja, Huang, and Yan (2011).
[w]Lee et al. (2014).
[x]Ólafsdóttir, Lauzon, Martinsdottir, and Kristbergsson (2006)
[y]Baranyi and Tamplin (2004).
Source: Adapted and modified from McDonald and Sun (1999) with permission.

using square root, gamma, and cardinal methods. Applications include modelling microbial growth or inactivation interfaces, the length of the lag phase for microorganisms in formulated ready-to-eat foods, and the production of microbial toxins (Whiting, 1995). More recently, artificial neural networks have also been used for modelling purposes (Marini, 2009). Some examples of secondary models used are given in Table 12.1.

Tertiary mathematical model development involves expressing secondary model predictions through a primary model. This is normally done with either commercial spreadsheets or computer software. Computer software programs provide an interface between the model's mathematics and the end user, allowing the information to be entered into the model, and then the prediction is viewed as a figure or table. However, tertiary-level mathematical modelling needs skilled personnel to interpret the information given by the models. The user should know the limits and conditions of the model's application to avoid flawed predictions. There are various types of the model software packages such as the USDA Pathogen Modeling Program, Growth Predictor, and *Pseudomonas* Predictor, which are used for mathematical modelling. Some examples of tertiary models used are given in Table 12.1.

The Institute of Food Research, UK, has produced Growth Predictor (http://www.ifr.ac.uk/Safety/GrowthPredictor/default.html), a model for predicting the growth of bacteria in microbiological broth as a function of temperature, pH, water activity, carbon dioxide, and acetic acid. The user can also input the inoculum level and a value for the physiological state of the bacteria. These factors allow the user control over the prediction of the lag phase.

The Pathogen Modeling Program was developed by the Food Safety Research Unit of the U.S. Department of Agriculture Research Services and can be downloaded at http://ars.usda.gov/Services/docs.htm?docid=6786. This model can be used to predict the growth, survival, inactivation, and toxin production of food-borne bacteria, primarily pathogens under various environmental conditions. This model allows users to input conditions for a variety of intrinsic and extrinsic environmental conditions, such as pH, temperature, water activity, atmosphere, concentrations of various acidulants, and irradiation dose. The program contains growth models for certain bacterial pathogens (*Clostridium perfringens*, *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, etc.) and specific environments (culture medium, food, etc.).

Microfit software, developed by the Institute of Food Research, UK, enables the estimation of growth rate, doubling time, initial cell number, and lag time from challenge test data. The software facilitates the comparison of individual growth curves with predictions from software packages such as Food Micro Model and the Pathogen Modeling Program.

The Seafood Spoilage and Safety Predictor software package was developed as a time/temperature integration device that facilitates the use of various mathematical seafood spoilage models. The software package predicts the shelf life and growth of specific spoilage organisms in seafood under constant or fluctuating temperature conditions. This software was developed at the National Institute of Aquatic Resources, Technical University of Denmark.

ComBase (http://www.combase.cc/index.php/en/) is an international database mainly composed of thousands of microbial growth and survival curves. It is maintained by the ComBase Consortium, which is a collaboration between the Food Standards Agency and the Institute of Food Research (UK) and the USDA Agricultural Research Service and its Eastern Regional Research Center (USA).

SymPrevius software (http://www.symprevius.org/) was supported by the French Departments of Research and Agriculture. It is a decision-making tool intended for the food industry and its partners, consisting of growth interfaces, growth simulation, growth-curve fitting, thermal destruction simulation, and bacterial survival simulation. The software was developed from a database that linked food, bacteria, and environmental characteristics to the incidence and the behaviour of microorganisms.

12.5 Model development

Development and implementation of mathematical modelling involve a series of steps such as identifying the key factors, experimental design, data collection and analysis, model validation, etc.

12.5.1 Recognizing the key factors

There are a number of intrinsic, extrinsic, and implicit factors that affect the growth and survival of microorganisms in food. Generally, intrinsic factors are the characteristics of the food itself, for example, pH, water activity, preservatives. Extrinsic factors include the environment in which the food is stored, for example, temperature, relative humidity, gaseous atmosphere. Implicit factors are the characteristics of the microorganism itself and how it behaves in the presence of intrinsic and extrinsic factors. Several factors may affect the growth and survival of a microorganism present in food and it is important to consider these factors and include them in the model. The intended use of the model is the prime consideration when defining the controlling factors to be included.

12.5.2 Experimental design

The range of conditions over which the model is to function should be distinct because empirical models should not be applied beyond the area defined by the conditions used to generate the model (Blackburn, 2000). An experimental design in which these factors can be altered easily is requisite, although the heterogeneity of food makes their use difficult for the generation of data for modelling (Maxcy & Wallen, 1983). Generally, microbiological media are used because they consist of all the components that can be reproducibly modified under the required conditions. If the model is intended for a specific food, then the choice of factors may need to be more focused, for example, the addition of specific organic acid to include the effects related to the undissociated molecule (Blackburn, 2000).

The inoculum size, microbial strain, and culture conditions all affect the outcome of the data and subsequent predictions. The initial inoculum size in the experimental

media or food products under investigation is an important parameter to be considered in experimental design. Large inoculum size requires incorporation of a higher concentration of antimicrobial agents in experimental food to achieve the targeted response, such as complete growth inhibition, lowering the specific growth rate, and/or lag-phase extension of the food pathogens. Changes in inoculum density as well as the absolute number (rather than concentration) of total organisms present in the test system can affect the overall outcome. Silva-Angulo, Zanini, Rodrigo, Rosenthal, and Martinez (2014) estimated the impact of inoculum size on the antibacterial activity of carvacrol and observed that the inoculum size significantly affected the lag phase, which was shorter for the higher inoculum concentration. It has been found that the duration of the lag phase depends inversely on the inoculum size in culture media. However, this phenomenon was observed only under a restricted condition, which could be explained by an increase in the variation in individual cell lag time when cells are stressed.

The standard inoculum concentration chosen by the NCCLS is 5×10^5 CFU/ml of broth medium. A 5×10^5 CFU/ml inoculum provides an acceptable challenge dose for assessing the biological activity of antimicrobial agents and is large enough to provide statistically satisfactory data for determining a minimum bactericidal concentration end point. If the inoculum is too small, significant bacterial resistance may not be detected (Peterson & Shanholtzer, 1992). All strains have diverse phenotypic responses so the type of strain selection and screening need to be carried out for modelling. Microbial growth conditions and storage conditions, including temperature and culture medium, can affect the microorganism's responses, so the likely conditions for the organism should be carefully selected. Furthermore, sampling time is one of the important considerations for experimental design and these should be focused around the most active phase of bacterial growth, such as the log phase (Blackburn & de, 2000). Because bacterial cells are actively metabolizing during this stage, they are particularly vulnerable to antimicrobial agents.

12.5.3 Data collection and analysis

Large amounts of experimental data are required to predict the effects of preservatives on growth, survival, or inactivation of microorganisms. Such data have often been generated using liquid laboratory media. Quantification of microorganisms at selected time points is usually by standard colony count methods, but automated methods such as absorbance or conductance measurements can be used to facilitate the generation of data in liquid media. In some studies, to convert the optical density to log CFU/ml, a relationship between the optical density at 600 nm and the viable count was established for the test bacteria (Gupta et al., 2012; Jaiswal, Gupta, Abu-Ghannam, & Cox, 2011).

The next step involves mathematical analysis of the data to produce a model. The aim of the model-fitting procedure is to find those values of the parameters that best describe the data by minimizing the sum of the squares of the differences between the model-simulated and the experimental values. However, this step is largely focused on the type of model to be utilized for modelling the data. For example, in the cases of

modified Gompertz, logistic model, and Baranyi model, a plot of microbial count versus time for each antimicrobial concentration was used to calculate the initial values for the parameters N_0 and N_{max} for the models evaluated (Gupta et al., 2012). However, advancements in computational software have simplified the implementation of complex mathematical models, which is less time-consuming and can be used to fit the microbial growth/survival curve. Several such softwares are available to the research community, such as Statgraphics Centurion and Design-Expert software. For the Baranyi model a program (DMFit) implemented in Microsoft Excel is used for fitting bacterial curves (DMFit; Institute of Food Research, Norwich, UK). The USDA Agricultural Research Service has developed a user-friendly, comprehensive data analysis tool, the Integrated Pathogen Modeling Model 2013. This tool allows users, without detailed programming knowledge, to analyse experimental kinetic data and fit the data to known mathematical models commonly used in predictive microbiology (Huang, 2014).

12.5.4 Validation of mathematical models

One of the most important aspects of model development is ensuring reliability of the models. The assessment of the reliability of mathematical models is possible only through the combination of several statistical analyses and proper investigation regarding the purposes for which the mathematical model was initially conceptualized and developed.

Fakruddin et al. (2011) suggested a two-step validation of a model once it has been developed. The first step is to test its accuracy with the new experimental data and new combinations of variables to determine if the model can describe the experimental data sufficiently. This is called internal validation or 'curve fitting'. This will allow an estimation of the goodness of fit and will show if and where additional data are needed. Complex models tend to be very specific, which can be a limitation when testing new data. The quality of fit of a mathematical model can be expressed as the determination of coefficient (R^2), which is an overall measure to evaluate the prediction obtained. The higher the value ($0 < R^2 < 1$) is, the better is the prediction by the model.

The second step is to compare model predictions with microbial responses in actual foods. This is called external validation. This will show the model's limitations and may show if additional factors must be tested and included in the model. Errors in growth or survival should always tend towards faster growth rates or better survival, respectively, to make a conservative prediction (Whiting, 1995).

Statistical measures such as mean square error (MSE) or root mean square error (RMSE) are commonly used to evaluate the difference between the model-estimated data and the experimental data. The model with the lower value of MSE or RMSE ($0 <$ MSE/RMSE) is more accurate at predicting (Brocklehurst, 2003; Zhao et al., 2014). MSE (Eqn (12.12)) can be defined by the following expression:

$$\text{MSE} = \frac{\sum (\text{Predicted} - \text{Observed})^2}{n - p} \tag{12.12}$$

RMSE (Eqn (12.13)) can be defined by the expression

$$\text{RMSE} = \sqrt{\frac{\sum (\text{Predicted} - \text{Observed})^2}{n - p}} \tag{12.13}$$

where n is the number of observations and p is the number of parameters to be estimated.

Some authors also suggest the application of bias and accuracy factors to validate the model (Ross, 1996). The accuracy factor indicates the average deviation between the model predictions and the observed results (Brocklehurst, 2003; Neumeyer, Ross, Thomson, & McMeekin, 1997). The accuracy factor (Eqn (12.14)) is defined by the expression

$$\text{Accuracy factor} = 10^{\frac{\sum \log\left(\frac{\text{Predicted}}{\text{Observed}}\right)}{n}} \tag{12.14}$$

where n is the number of observations; 'predicted' is a parameter under consideration such as the model-predicted generation time; and 'observed' is the experimentally observed generation time or specific growth rate or lag phase. The higher the value of the accuracy factor, the lower in precision is the average estimate. An accuracy factor of 2 indicates that the prediction is on average different from the observed value by a factor of 2 (Ross, 1996).

The bias factor is used to indicate the structural deviations of a model (Zhao et al., 2014). It describes the observed values that lie above or below the line of equivalence and, if so, by how much. A bias factor >1 indicates a 'fail-safe' model (Brocklehurst, 2003; Zhao et al., 2014; Zhou et al., 2008). Bias factor (Eqn (12.15)) can be defined by the following expression:

$$\text{Bias factor} = \frac{\sum \log\left(\frac{\text{Observed}}{\text{Predicted}}\right)}{n} \tag{12.15}$$

12.6 Modelling the effects of natural antimicrobial agents

Generally, the models that can be used for describing the kinetics of survival curves are either empirical or based on biological assumptions (Gupta et al., 2012). Primary mathematical models such as Gompertz or modified Gompertz, logistic, and Baranyi-Roberts are the most commonly used mathematical models to analyse the delay or inhibition of the growth of organisms (Belda-Galbis et al., 2014; Gupta et al., 2012; Jaiswal et al., 2011; Koutsoumanis, Lambropoulou, & Nychas, 1999; Pina-Pérez, Silva-Angulo, Rodrigo, & Martínez-López, 2009; Tornuk, Ozturk, Sagdic, Yilmaz,

& Erkmen, 2014; Velázquez-Nuñez, Avila-Sosa, Palou, & López-Malo, 2013; Yao, Tan, Zhang, Su, & Wei, 1998). However, some authors have also used the fuzzy logic system and artificial neural network to model the antimicrobial activity of natural food preservatives (Sagdic, Ozturk, & Kisi, 2012).

12.6.1 Plant extracts

Plant extracts are rich in phenolic compounds and other secondary metabolites and some have antimicrobial activity. Several studies have been carried out to find new sources of antimicrobial agents from plant parts that can be used as alternatives to synthetic preservatives (Jaiswal et al., 2011; Pina-Pérez et al., 2009; Tornuk et al., 2014). Yao et al. (1998) monitored the disturbances in *Proteus rettgeri* growth using the acoustic wave bacterial growth sensor in aqueous extracts of various tea samples, such as green tea, Fuzhuan brick tea, Oolong tea, Kudin tea, and black tea. The modified Gompertz model was successfully applied to fit the growth kinetic concentration curves of *P. rettgeri*, and the kinetic parameters such as asymptote, maximum specific growth rate, lag time, and generation time were accurately estimated by using the growth response model, and these kinetic parameters were used to characterize the antimicrobial properties of tea.

Jaiswal et al. (2011) evaluated the antibacterial activity of York cabbage against several food pathogens, and the survival curve was mathematically modelled using the Baranyi model. The authors observed that cabbage extracts had an antagonizing effect on the selected food pathogens, showing a remarkable dose−response relationship with an increase in the lag-phase duration and decrease in the maximum specific growth rate. Analyses of variance indicated that the maximum specific growth rate was significantly reduced with increasing extract concentration. The Baranyi model was capable of fitting the experimental data very reasonably and produced curves with an R^2 value ranging from 87.5% to 99.6%. Pina-Pérez et al. (2009) conducted an experiment to extend the shelf life and enhance the safety of liquid whole egg/skim milk mixed beverages. The antibacterial activity of cocoa powder (CocoaNOX 12%®, Natraceutical S.A., Valencia, Spain) was estimated against *B. cereus*, which was inoculated into skim milk and liquid whole egg/skim milk mixed beverages. The beverages were also treated with a pulsed electric field in the presence and absence of cocoa powder. The kinetic results were modelled with the Bigelow model, Weibull distribution function, modified Gompertz equation, and log−logistic models. It was reported that beverages supplemented with the antimicrobial compound showed higher inactivation levels, reaching a 3.30 $\log_{10}$ cycle reduction compared to pulsed electric field-treated samples under the same conditions. Among the models fitted, the four-parameter log−logistic model showed the best fit for all beverages.

When estimating the inhibitory effects of bay leaf, rosemary, sage, and thyme hydrosols on the growth of *Staphylococcus aureus* inoculated onto fresh-cut apples, Tornuk et al. (2014) modelled the bacterial growth using modified Gompertz, logistic, Richards, Stannard, and Whiting and Buchanan to describe the inactivation of the bacterium. Validation of the mathematical models was carried out using various statistical

parameters such as mean percentage error, mean bias error, RMSE and determination of coefficient. The modified Gompertz, logistic, and Stannard models exhibited better fits than the Richards and Whiting and Buchanan models regarding these statistical parameters.

In other studies, Belda-Galbis et al. (2014) estimated the antibacterial activity of *Stevia rebaudiana* Bertoni against *Listeria innocua* in a medium supplemented with a leaf infusion, a crude extract, and a purified extract; experimental data were fitted to the modified Gompertz model. Antibacterial activity of *Stevia* was determined based on the lag-phase duration and the maximum specific growth rate reached. These authors did not observe a significant difference between the controls and the samples containing the purified extract; therefore no bacteriostatic/bactericidal effect against *L. innocua* was documented. However, an increase in *lag phase* and a decrease in maximum specific growth rate were found for the crude extract and the infusion. The crude extract was able to multiply lag time by twofold. With the infusion, the lag time value was 10 times higher than the lag time value without *Stevia*. Maximum specific growth rate was halved in both cases, both in the presence of the crude extract and in the presence of the infusion. Consequently, a statistically significant antimicrobial effect can be attributed to these *Stevia* extracts.

12.6.2 Essential oils

Essential oils are aromatic oily liquids obtained from plant material such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots. Several studies were carried out to exploit the potential of essential oils as a source of natural antimicrobial agents. Koutsoumanis et al. (1999) estimated the effects of oregano essential oil under various temperature and pH profiles on tarama salad, a traditional Greek appetizer, against *Salmonella* serotype Enteritidis. For each combination of the environmental factors, the bacterial counts were modelled as a function of time to estimate the kinetic parameters of the *S.* Enteritidis. Several inactivation curves representing various combinations of essential oil concentration, pH, and storage temperature were generated using the Baranyi model and the modified Gompertz equation. Comparing the outputs of the two models, these authors reported that no lag phase was derived from the Baranyi model. In contrast, the Gompertz equation gave a short lag phase in most cases (Figure 12.2). The comparative results obtained from the Baranyi and Gompertz models indicate that the Baranyi model, which is generally used for fitting growth data, may also be useful as an alternative model for describing the inactivation of bacteria under suboptimal environments such as the addition of natural antimicrobial systems. In other studies, Skandamis and Nychas (2000) evaluated the antibacterial activity of oregano essential oil at various storage temperatures and pH against *E. coli* O157:H7 NCTC 12,900 in homemade eggplant salad (a traditional Greek appetizer). For each combination of environmental factors, the bacterial counts were modelled, using the Baranyi model, as a function of time to estimate the kinetic parameters of the pathogen.

Similar studies were also carried out by Portillo-Ruiz, Sánchez, Ramos, Muñoz, and Nevárez-Moorillón (2012); these authors evaluated the antifungal effect of

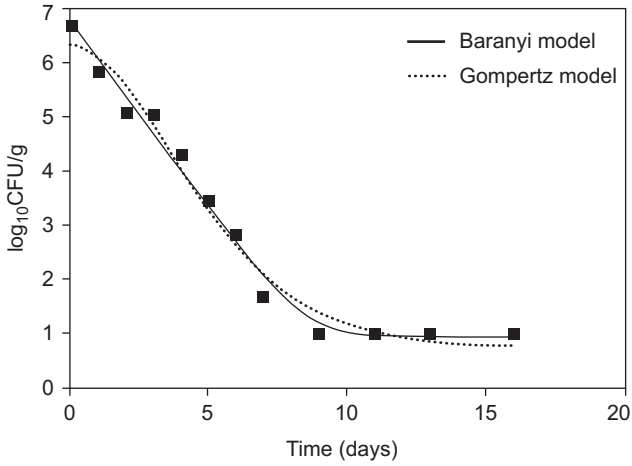

Figure 12.2 Inactivation curves for *Salmonella enteritidis*, fitted with the Baranyi and Gompertz models, in homemade tarama salad with initial pH 4.3, supplemented with 0.5% oregano essential oil, and stored at 20 °C.
Source: Adapted from Koutsoumanis et al. (1999) with permission.

Mexican oregano (*Lippia berlandieri* Schauer) essential oil fractions on the growth of *Aspergillus*, *Penicillium*, and *Rhizopus* spp., and they fitted the growth curves using the modified Gompertz model. They reported that Mexican oregano essential oils showed a linear reduction in specific growth rate, on the maximum mould growth at the stationary phase, and an increase in the lag time as the concentration of the oregano essential oils increased. Velázquez-Nuñez et al. (2013) compared the antifungal efficacy of orange peel essential oils, applied either by vapour exposure or by direct addition, on the growth of *Aspergillus flavus*; they calculated the radial growth rate and lag phase using the Gompertz equation. Significant differences in modified Gompertz model parameters were observed in both methods studied. Maximum mould growth and maximum specific growth rate showed that increasing the concentration of orange peel essential oils decreases these parameters.

Belda-Galbis, Pina-Pérez, Leufvén, Martínez, and Rodrigo (2013) estimated the effects of carvacrol (an important component of oregano oil) and citral (present in the essential oils of several plants such as lemon grass) at various storage temperatures against *E. coli* O157:H7 and *L. monocytogenes* surrogates. The results were integrated into a stochastic model to perform probabilistic predictions of the final microbial load. The study takes into account the variability in growth conditions by means of maximum growth rate and lag-time duration kinetic parameters, using the Monte Carlo simulation. It was reported that for each strain, regardless of temperature, both lag phase and maximum specific growth rate proved to be dependent on the antibacterial concentration at noninhibitory doses: the higher the concentration, the lower the maximum specific growth rate. Consequently, both carvacrol and citral showed a bacteriostatic effect on *E. coli* K12 and *L. innocua* growth in the temperature range studied. Figure 12.3 shows the lag phase and maximum specific growth

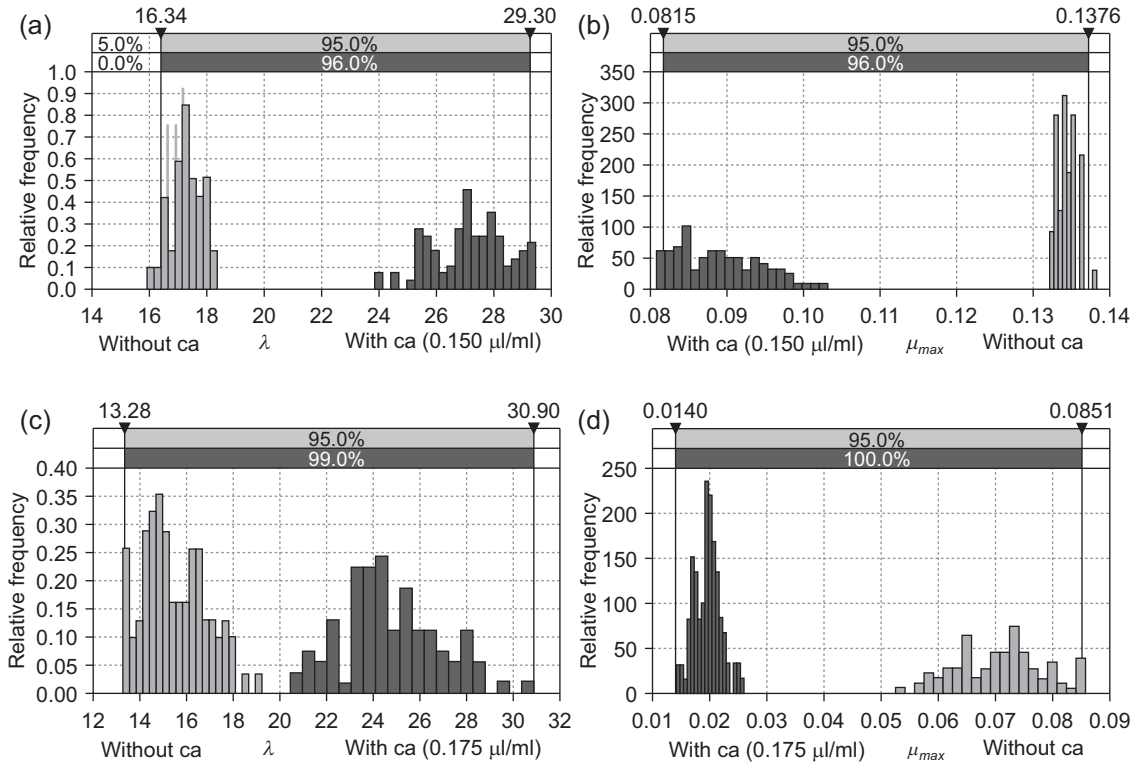

Figure 12.3 Growth rate (μ_{max}; ($\log_{10}$ (CFU/ml))/h) and lag time (λ; h) distributions obtained from Monte Carlo simulation for *Escherichia coli* K12 (a and b) and *Listeria innocua* (c and d) grown at 15 °C, with and without carvacrol (ca).
Source: Adapted from Belda-Galbis et al. (2013) with permission.

rate distributions obtained from the Monte Carlo simulation for the two bacteria in the absence and in the presence of carvacrol. As can be seen in the figure, in general terms, the antimicrobial addition increases the dispersion of the parameters' distributions. These results indicate that stress conditions, such as exposure to natural antimicrobials, lead to significant changes in the shape, mean, and variance of lag-phase distributions.

Muñoz, Guevara, Palop, and Fernández (2010) applied a stochastic approach to evaluate the growth of heat-damaged *L. monocytogenes* cells as influenced by pH and the presence of eugenol (essential oil found in cloves) using an individual-based approach to growth through OD measurements. Both the lag-phase duration and the h_0 parameter were derived from the growth curves obtained. Histograms showed a shift to longer lag phases and an increase in variability with high pH and the presence of eugenol. The authors observed that both Monte Carlo and regression analyses gave a good indication of the probability of a certain level of growth. In a similar study carried out by Silva-Angulo et al. (2014), the growth kinetics of *L. innocua* and *L. monocytogenes* serovar 4b were compared in the presence of carvacrol, and the growth curves were fitted using the modified Gompertz model, and the lag phase and maximum growth rate were calculated (Figure 12.4). It was reported that the increase in carvacrol concentration resulted in an extended lag phase and lower maximum growth rate in comparison with the untreated cells. In the presence of carvacrol, the lower inoculum size showed an increased growth rate and relatively longer lag phase compared to the higher inoculum size.

12.6.3 Seaweed extracts

Marine macroalgae, commonly known as seaweeds, are potentially renewable resources in the marine habitat. Seaweeds are rich in several secondary metabolites, such as polyphenols (phlorotannins, fucoxanthin, flavonoids) and polysaccharides (fucoidan, laminarans). Studies show that they are good sources of antimicrobial agents. Gupta et al. (2012) used brown seaweed extract as a natural antimicrobial agent against several bacteria, and growth kinetics were modelled using Baranyi, modified Gompertz, and logistic models for describing the survival of organisms in the presence of various concentrations of the extract. The authors observed that in most of the cases, the R^2 values for all the models were greater than 90%, representing a good fit to the experimental data. All the parameters obtained for the three mathematical models were directly related to the extract concentration. Analyses of variance indicated that the maximum specific growth rate was significantly reduced (99−96.8%), whereas the lag phase for all three models increased (20−81%) with the extract concentration. These authors observed that all three models were capable of fitting the experimental data very rationally and produced almost similar curves; however, no model could consistently produce the best fit to all the growth curves. The models were statistically validated with the use of the F test. The calculated F values were lower than the F table values, indicating that there was no significant difference in the goodness of fit between the three models.

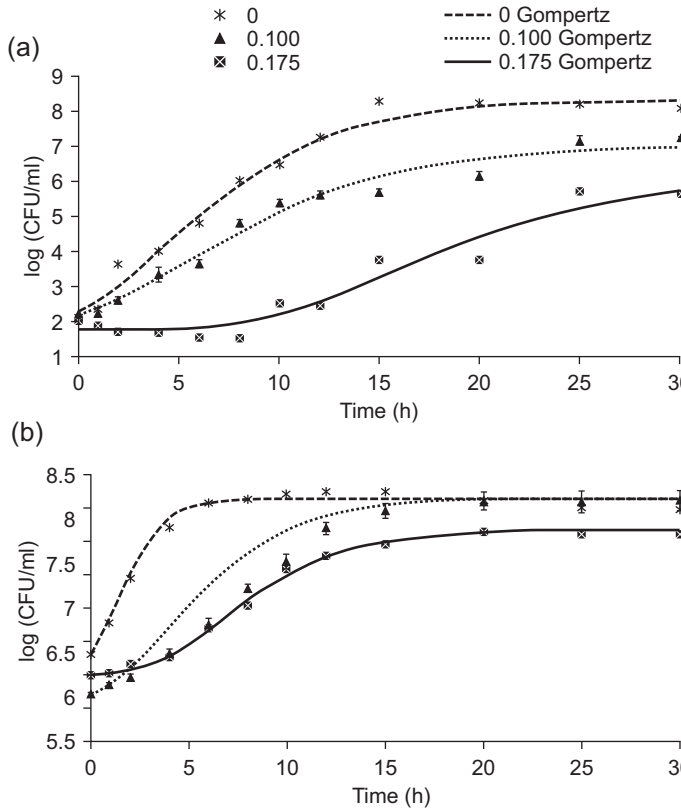

Figure 12.4 (a) *Listeria innocua* and (b) *Listeria monocytogenes* growth curves in the presence of different concentrations of carvacrol (μl/ml) in reference medium. The lines represent the fit of experimental data to the modified Gompertz model. The standard deviation associated with each average value is expressed by error bars.
Source: Adapted from Silva-Angulo et al. (2014) with permission.

12.6.4 Others

Guerrero, Tognon, and Alzamora (2005) evaluated the resistance of *Saccharomyces cerevisiae* to the action of ultrasound at 45 °C in Sabouraud broth containing 1000 ppm low-weight chitosan, and the experimental data were modelled using the modified Gompertz equation and Weibull distribution of resistances. These authors reported that addition of chitosan enhanced the inactivation by ultrasound. Lavermicocca, Valerio, and Visconti (2003) estimated the fungicidal activity of phenyllactic acid (organic acid, produced by many microorganisms, especially lactic acid bacteria) against 23 fungal strains belonging to 14 species of *Aspergillus*, *Penicillium*, and *Fusarium* that were isolated from bakery products, flours, or cereals. Optical density was recorded every 24 h from time 0 to 120 h and was used to generate growth curves for each fungal strain; the Gompertz model was used as a mathematical means of fitting growth curves to estimate microbial growth kinetics.

To estimate the effect of interaction between two strains of *Lactobacillus plantarum* and two food-borne pathogens, *L. monocytogenes* and *E. coli*, Aguilar, Vanegas, and Klotz (2011) used whole Ultra-high Temperature (UHT) processed milk as an experimental medium at 37 °C. To determine the type of interaction between the two bacterial populations in cocultures and to evaluate the antagonistic activity of the lactic acid bacteria on the pathogenic bacteria, the growth curves, the kinetic parameters, and the pH profiles of mono- and cocultures were compared. These authors reported that the lactic acid bacteria reduced the growth of *E. coli* and of *L. monocytogenes* by 4.0 and ~5.0 $\log_{10}$ cycles, respectively, and influenced other growth kinetic parameters, such as maximum specific growth rate and lag phase, in the different binary combinations. Pena and de Massaguer (2006) evaluated the adaptation time of *Alicyclobacillus acidoterrestris* CRA 7152 in orange juice, which was determined as a response to pH, temperature, soluble solids, and nisin (polycyclic antibacterial peptide, produced by lactic acid bacteria, especially *Lactococcus lactis*) addition. Results showed that the Baranyi and Roberts model was better than the modified Gompertz model, considering the determination of coefficient (R^2) as a measure to evaluate the prediction obtained.

Zhou et al. (2013) evaluated the inhibition effects of vapour-phase thymol, modified atmosphere (MA), and their combination against *Salmonella* spp. on raw shrimp. Lag time and maximum growth rate of *Salmonella* spp. under each treatment were obtained using Baranyi and Roberts models. Combination treatment with vapour-phase thymol and MA exhibited greater inhibition effectiveness than each individual treatment and a synergistic antimicrobial effectiveness could be observed on the lag-time extension. The maximum lag time of *Salmonella* spp., at 12 °C, was extended 59.6% more by the combination treatment of 0.8 mg/l thymol + modified atmosphere (36.97 h) compared to 0.8 mg/l thymol treatment and MA treatment alone (23.16 h in total).

12.7 Conclusion and future trends

Predictions of microbial behaviour are not 100% accurate. Variations and uncertainty are introduced through experimental error, strain variation, and other intrinsic, extrinsic, and implicit factors that affect the growth and survival of the microorganism in the food. Efforts were made by mathematicians to develop new forms of models that provide more reliable estimates between model-simulated data and experimental data. Consequently, a plethora of growth models has been developed to model the growth, survival, inactivation, or biochemical process of food-borne organisms. However, primary mathematical models such as the modified Gompertz, logistic, and Baranyi-Roberts models are still the most commonly used models to analyse the delay or inhibition of growth of the organisms, and parameters normally include lag-phase duration, growth rate, and maximum population density.

Increased attention is being given to the quality and safety of food not only because of the consumer's perspective but also because of governmental pressure. Regulations by the U.S. FDA have required processors to achieve a 5.0 $\log_{10}$ reduction in the numbers of the most resistant pathogens in their finished products. The ruling has

accelerated the search for novel processing technologies, including application of preservatives from natural origin in food that can ensure product safety, yet maintain the desired nutritional and sensory characteristics. Consequently, most models are targeted towards elimination of food pathogens to help ensure microbiologically safe food products and, as a result, there is likely to be increased research in the area of food microbiology and the application of predictive modelling to ensure that their applied processes are up to the very highest standards.

References

Aguilar, C., Vanegas, C., & Klotz, B. (2011). Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. *Journal of Dairy Research*, *78*(2), 136−143.

Álvarez-Fernández, E., Cancelo, A., Díaz-Vega, C., Capita, R., & Alonso-Calleja, C. (2013). Antimicrobial resistance in *E. coli* isolates from conventionally and organically reared poultry: a comparison of agar disc diffusion and Sensi Test Gram-negative methods. *Food Control*, *30*(1), 227−234.

Andraud, M., Chauvin, C., Sanders, P., & Laurentie, M. (2011). Pharmacodynamic modeling of *in vitro* activity of marbofloxacin against *Escherichia coli* strains. *Antimicrobial Agents and Chemotherapy*, *55*(2), 756−761.

Arrhenius, S. (1889). About the reaction rate in the inversion of cane sugar by acids. *Journal of Physical Chemistry*, *4*, 226−248.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, *23*(3−4), 277−294.

Baranyi, J., Roberts, T., & McClure, P. (1993). A non-autonomous differential equation to modelbacterial growth. *Food Microbiology*, *10*(1), 43−59.

Baranyi, J., & Tamplin, M. L. (2004). ComBase: a common database on microbial responses to food environments. *Journal of Food Protection*, *67*(9), 1967−1971.

Belda-Galbis, C. M., Pina-Pérez, M. C., Espinosa, J., Marco-Celdrán, A., Martínez, A., & Rodrigo, D. (2014). Use of the modified Gompertz equation to assess the *Stevia rebaudiana* Bertoni antilisterial kinetics. *Food Microbiology*, *38*, 56−61.

Belda-Galbis, C. M., Pina-Pérez, M. C., Leufvén, A., Martínez, A., & Rodrigo, D. (2013). Impact assessment of carvacrol and citral effect on *Escherichia coli* K12 and *Listeria innocua* growth. *Food Control*, *33*(2), 536−544.

Blackburn, C. de W. (2000). Modelling shelf-life. In D. Kilcast, & P. Subramaniam (Eds.), *The stability and shelf-life of food* (pp. 54−78). Cambridge, London, UK: Woodhead.

Boekel, M. A. J. S. V. (1996). Statistical aspects of kinetic modeling for food science problems. *Journal of Food Science*, *61*(3), 477−486.

Botterweck, A. A. M., Verhagen, H., Goldbohm, R. A., Kleinjans, J., & van den Brandt, P. A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study. *Food and Chemical Toxicology*, *38*(7), 599−605.

Bovill, R., Bew, J., Cook, N., D'agostino, M., Wilkinson, N., & Baranyi, J. (2000). Predictions of growth for *Listeria monocytogenes* and *Salmonella* during fluctuating temperature. *International Journal of Food Microbiology*, *59*(3), 157−165.

Brocklehurst, T. (2003). Challenge of food and the environment. In R. C. McKellar, & X. Lu (Eds.), *Modeling microbial responses in food* (pp. 197−232). Boca Raton, FL: CRC Press.

Brul, S., & Coote, P. (1999). Preservative agents in foods: mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1−2), 1−17.

Buchanan, R. L. (1993). Predictive food microbiology. *Trends in Food Science & Technology*, *4*(1), 6−11.

Buchanan, R., Whiting, R., & Damert, W. (1997). When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food Microbiology*, *14*(4), 313−326.

Běhrádek, J. (1930). Temperature coefficients in biology. *Biological Reviews*, *5*(1), 30−58.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1−20.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*, *23*(2), 174−181.

Davey, K. (1993). Linear-Arrhenius models for bacterial growth and death and vitamin denaturations. *Journal of Industrial Microbiology*, *12*(3−5), 172−179.

Diao, W.-R., Hu, Q. P., Feng, S.-S., Li, W. Q., & Xu, J.-G. (2013). Chemical composition and antibacterial activity of the essential oil from green huajiao (*Zanthoxylum schinifolium*) against selected foodborne pathogens. *Journal of Agricultural and Food Chemistry*, *61*(25), 6044−6049.

Draper, N. R. (1988). Response surface designs. In *Encyclopaedia of statistical sciences*. New York: Wiley.

Fakruddin, M., Mazumdar, R. M., & Mannan, K. S. B. (2011). Predictive microbiology: modeling microbial responses in food. *Ceylon Journal of Science (Biological Sciences)*, *40*(2), 121−131.

Gibson, A. M., Bratchell, N., & Roberts, T. (1987). The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. *Journal of Applied Microbiology*, *62*(6), 479−490.

Gibson, A. M., & Hocking, A. D. (1997). Advances in the predictive modelling of fungal growth in food. *Trends in Food Science & Technology*, *8*(11), 353−358.

Gonzales-Barron, U. A. (2011). Predictive microbial modelling. In D.-W. Sun (Ed.), *Handbook of food safety engineering* (pp. 108−152). Oxford, UK: Wiley-Blackwell.

Guerrero, S., Tognon, M., & Alzamora, S. M. (2005). Response of Saccharomyces cerevisiae to the combined action of ultrasound and low weight chitosan. *Food Control*, *16*(2), 131−139.

Gupta, S., Cox, S., Rajauria, G., Jaiswal, A. K., & Abu-Ghannam, N. (2012). Growth inhibition of common food spoilage and pathogenic microorganisms in the presence of brown seaweed extracts. *Food and Bioprocess Technology*, *5*(5), 1907−1916.

Hauschild, A. (1982). Assessment of botulism hazards from cured meat products. *Food Technology*, *36*(12), 95−104.

Hayes, J., Stepanyan, V., Allen, P., O'Grady, M., & Kerry, J. (2010). Effect of lutein, sesamol, ellagic acid and olive leaf extract on the quality and shelf-life stability of packaged raw minced beef patties. *Meat Science*, *84*(4), 613−620.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*, *22*(4), 273−292.

Houtsma, P., Kant-Muermans, M., Rombouts, F., & Zwietering, M. (1996). Model for the combined effects of temperature, pH, and sodium lactate on growth rates of *Listeria innocua* in broth and Bologna-type sausages. *Applied and Environmental Microbiology*, *62*(5), 1616−1622.

Huang, L. (2014). IPMP 2013 − A comprehensive data analysis tool for predictive microbiology. *International Journal of Food Microbiology*, *171*(0), 100−107.

Isabelle, L., & André, L. (2006). Quantitative prediction of microbial behaviour during food processing using an integrated modelling approach: a review. *International Journal of Refrigeration*, *29*(6), 968−984.

Jaiswal, A. K., Gupta, S., Abu-Ghannam, N., & Cox, S. (2011). Application of Baranyi function to model the antibacterial properties of solvent extract from Irish York cabbage against food spoilage and pathogenic bacteria. *Food Science and Technology International*, *17*(5), 495−502.

Jaiswal, A. K., Rajauria, G., Abu-Ghannam, N., & Gupta, S. (2011). Phenolic composition, antioxidant capacity and antibacterial activity of selected Irish Brassica vegetables. *Natural Product Communications*, *6*(9), 1299−1304.

Jaiswal, S., Varma, P., O'Neill, L., Duffy, B., & McHale, P. (2013). An investigation of the biochemical properties of tetrazines as potential coating additives. *Materials Science and Engineering, C: Materials for Biological Applications*, *33*(4), 1925−1934.

Jason, A. (1983). A deterministic model for monophasic growth of batch cultures of bacteria. *Antonie Van Leeuwenhoek*, *49*(6), 513−536.

Jordán, M. J., Lax, V., Rota, M. C., Lorán, S., & Sotomayor, J. A. (2013). Effect of bioclimatic area on the essential oil composition and antibacterial activity of *Rosmarinus officinalis* L. *Food Control*, *30*(2), 463−468.

Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel natural food antimicrobials. *Annual Review of Food Science and Technology*, *3*, 381−403.

Juneja, V., Eblen, B., & Ransom, G. (2001). Thermal inactivation of *Salmonella* spp. in Chicken broth, beef, pork, turkey, and chicken: determination of D-and Z-values. *Journal of Food Science*, *66*(1), 146−152.

Juneja, V. K., Huang, L., & Yan, X. (2011). Thermal inactivation of foodborne pathogens and the USDA pathogen modeling program. *Journal of Thermal Analysis and Calorimetry*, *106*(1), 191−198.

Juneja, V. K., Snyder, O. P., Jr., & Marmer, B. S. (1997). Thermal destruction of *Escherichia coli* O157: H7 in beef and chicken: determination of D- and Z-values. *International Journal of Food Microbiology*, *35*(3), 231−237.

Khuri, A. I. (2011). Response surface methodology. In *International encyclopedia of statistical science* (pp. 1229−1231). Springer.

Kim, S.-J., Cho, A. R., & Han, J. (2013). Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control*, *29*(1), 112−120.

Koutsoumanis, K., Lambropoulou, K., & Nychas, G. (1999). A predictive model for the non-thermal inactivation of *Salmonella enteritidis* in a food model system supplemented with a natural antimicrobial. *International Journal of Food Microbiology*, *49*(1), 63−74.

Krist, K. A., Ross, T., & McMeekin, T. A. (1998). Final optical density and growth rate; effects of temperature and NaCl differ from acidity. *International Journal of Food Microbiology*, *43*(3), 195−203.

Lambert, R. J. W., & Lambert, R. (2003). A model for the efficacy of combined inhibitors. *Journal of Applied Microbiology*, *95*(4), 734−743.

Lambert, R., & Pearson, J. (2000). Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. *Journal of Applied Microbiology*, *88*(5), 784−790.

Lavermicocca, P., Valerio, F., & Visconti, A. (2003). Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Applied and Environmental Microbiology*, *69*(1), 634−640.

Lee, Y. J., Jung, B. S., Yoon, H. J., Kim, K.-T., Paik, H.-D., & Lee, J.-Y. (2014). Predictive model for the growth kinetics of *Listeria monocytogenes* in raw pork meat as a function of temperature. *Food Control*, *44*, 16−21.

Marini, F. (2009). Artificial neural networks in foodstuff analyses: trends and perspectives a review. *Analytica Chimica Acta*, *635*(2), 121−131.

Maxcy, R., & Wallen, S. (1983). Heterogeneity of samples as a problem in shelf-life prediction. *Journal of Food Protection*, *46*, 542−544.

McDonald, K., & Sun, D.-W. (1999). Predictive food microbiology for the meat industry: a review. *International Journal of Food Microbiology*, *52*(1), 1−27.

McMeekin, T. A., Olley, J., & Ross, T. (1993). *Predictive microbiology: theory and application*. Taunton, UK: Research Studies Press.

Monod, J. (1949). The growth of bacterial cultures. *Annual Reviews in Microbiology*, *3*(1), 371−394.

Mueller, M., de la Peña, A., & Derendorf, H. (2004). Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. *Antimicrobial Agents and Chemotherapy*, *48*(2), 369−377.

Muñoz, M., Guevara, L., Palop, A., & Fernández, P. S. (2010). Prediction of time to growth of *Listeria monocytogenes* using Monte Carlo simulation or regression analysis, influenced by sublethal heat and recovery conditions. *Food Microbiology*, *27*(4), 468−475.

Neumeyer, K., Ross, T., Thomson, G., & McMeekin, T. (1997). Validation of a model describing the effects of temperature and water activity on the growth of psychrotrophic pseudomonads. *International Journal of Food Microbiology*, *38*(1), 55−63.

Ólafsdóttir, G., Lauzon, H., Martinsdottir, E., & Kristbergsson, K. (2006). Influence of storage temperature on microbial spoilage characteristics of haddock fillets (*Melanogrammus aeglefinus*) evaluated by multivariate quality prediction. *International Journal of Food Microbiology*, *111*(2), 112−125.

Pena, W. E. L., & de Massaguer, P. R. (2006). Microbial modeling of *Alicyclobacillus acidoterrestris* CRA 7152 growth in orange juice with nisin added. *Journal of Food Protection*, *69*(8), 1904−1912.

Peterson, L., & Shanholtzer, C. (1992). Tests for bactericidal effects of antimicrobial agents: technical performance and clinical relevance. *Clinical Microbiology Reviews*, *5*(4), 420−432.

Pina-Pérez, M., Silva-Angulo, A., Rodrigo, D., & Martínez-López, A. (2009). Synergistic effect of Pulsed Electric Fields and CocoanOX 12% on the inactivation kinetics of Bacillus cereus in a mixed beverage of liquid whole egg and skim milk. *International Journal of Food Microbiology*, *130*(3), 196−204.

Portillo-Ruiz, M. C., Sánchez, R. A.-S., Ramos, S. V., Muñoz, J. V. T., & Nevárez-Moorillón, G. V. (2012). Antifungal effect of Mexican oregano (*Lippia berlandieri* Schauer) essential oil on a wheat flour-based medium. *Journal of Food Science*, *77*(8), M441−M445.

Ratkowsky, D., Olley, J., McMeekin, T., & Ball, A. (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology*, *149*(1), 1−5.

Ratkowsky, D., Ross, T., McMeekin, T., & Olley, J. (1991). Comparison of Arrhenius-type and Bêlehrádek-type models for prediction of bacterial growth in foods. *Journal of Applied Microbiology*, *71*(5), 452−459.

Richards, F. (1959). A flexible growth function for empirical use. *Journal of Experimental Botany*, *10*(2), 290−301.

Roberts, T. (1989). Combinations of antimicrobials and processing methods. *Food Technology*, *43*, 156−163.

Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Microbiology*, *81*(5), 501−508.

Ross, T., & McMeekin, T. (1995). Predictive microbiology and HACCP. In A. M. Pearson, & T. R. Dutson (Eds.), *HACCP in meat, poultry, and fish processing* (pp. 330−357). Springer.

Sagdic, O., Ozturk, I., & Kisi, O. (2012). Modeling antimicrobial effect of different grape pomace and extracts on *S. aureus* and *E. coli* in vegetable soup using artificial neural network and fuzzy logic system. *Expert Systems with Applications*, *39*(8), 6792−6798.

Schnute, J. (1981). A versatile growth model with statistically stable parameters. *Canadian Journal of Fisheries and Aquatic Sciences*, *38*(9), 1128−1140.

Silva-Angulo, A. B., Zanini, S. F., Rodrigo, D., Rosenthal, A., & Martinez, A. (2014). Growth kinetics of *Listeria innocua* and *Listeria monocytogenes* under exposure to carvacrol and the occurrence of sublethal damage. *Food Control*, *37*(1), 336−342.

Skandamis, P. N., & Nychas, G. J. E. (2000). Development and evaluation of a model predicting the survival of *Escherichia coli* O157: H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Applied and Environmental Microbiology*, *66*(4), 1646−1653.

Solórzano-Santos, F., & Miranda-Novales, M. G. (2012). Essential oils from aromatic herbs as antimicrobial agents. *Current Opinion in Biotechnology*, *23*(2), 136−141.

Stannard, C. J., Williams, A. P., & Gibbs, P. A. (1985). Temperature/growth relationships for psychrotrophic food-spoilage bacteria. *Food Microbiology*, *2*(2), 115−122.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987−6000.

Tornuk, F., Ozturk, I., Sagdic, O., Yilmaz, A., & Erkmen, O. (2014). Application of predictive inactivation models to evaluate survival of *Staphylococcus aureus* in fresh-cut apples treated with different plant hydrosols. *International Journal of Food Properties*, *17*(3), 587−598.

Van Impe, J., Nicolaï, B. M., Martens, T., De Baerdemaeker, J., & Vandewalle, J. (1992). Dynamic mathematical model to predict microbial growth and inactivation during food processing. *Applied and Environmental Microbiology*, *58*(9), 2901−2909.

Van Impe, J. F., Nicolaï, B. M., Schellekens, M., Martens, T., & De Baerdemaeker, J. (1995). Predictive microbiology in a dynamic environment: a system theory approach. *International Journal of Food Microbiology*, *25*(3), 227−249.

Vásconez, M. B., Flores, S. K., Campos, C. A., Alvarado, J., & Gerschenson, L. N. (2009). Antimicrobial activity and physical properties of chitosan−tapioca starch based edible films and coatings. *Food Research International*, *42*(7), 762−769.

Velázquez-Nuñez, M. J., Avila-Sosa, R., Palou, E., & López-Malo, A. (2013). Antifungal activity of orange (*Citrus sinensis* var. Valencia) peel essential oil applied by direct addition or vapor contact. *Food Control*, *31*(1), 1−4.

Whiting, R. C. (1995). Microbial modeling in foods. *Critical Reviews in Food Science & Nutrition*, *35*(6), 467−494.

Whiting, R., & Buchanan, R. (1993). A classification of models in predictive microbiology-reply. *Food Microbiology*, *10*(2), 175−177.

Whiting, R., & Buchanan, R. (1994). *Scientific status summary: microbial modeling*. USA: Food Technology.

Whiting, R., & Cygnarowicz-Provost, M. (1992). A quantitative model for bacterial growth and decline. *Food Microbiology*, *9*(4), 269−277.

Williams, M. L., Landel, R. F., & Ferry, J. D. (1955). The temperature dependence of relaxation mechanisms in amorphous polymers and other glass-forming liquids. *Journal of the American Chemical Society*, *77*(14), 3701−3707.

Xiong, R., Xie, G., Edmondson, A., & Sheard, M. (1999). A mathematical model for bacterial inactivation. *International Journal of Food Microbiology*, *46*(1), 45−55.

Yao, S., Tan, H., Zhang, H., Su, X., & Wei, W. (1998). Bulk acoustic wave bacterial growth sensor applied to analysis of antimicrobial properties of tea. *Biotechnology Progress*, *14*(4), 639−644.

Zhao, J., Gao, J., Chen, F., Ren, F., Dai, R., Liu, Y., et al. (2014). Modeling and predicting the effect of temperature on the growth of *Proteus mirabilis* in chicken. *Journal of Microbiological Methods*, *99*(0), 38−43.

Zhou, K., Cui, T., Li, P., Liang, N., Liu, S., Ma, C., et al. (2008). Modelling and predicting the effect of temperature, water activity and pH on growth of *Streptococcus iniae* in Tilapia. *Journal of Applied Microbiology*, *105*(6), 1956−1965.

Zhou, S., Sheen, S., Pang, Y. H., Liu, L., & Yam, K. L. (2013). Antimicrobial effects of vapor phase thymol, modified atmosphere, and their combination against *Salmonella* spp. on raw shrimp. *Journal of Food Science*, *78*(5), M725−M730.

Zwietering, M., Jongenburger, I., Rombouts, F., & Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875−1881.

Zwietering, M., Rombouts, F., & Van'T Riet, K. (1993). Some aspects of modelling microbial quality of food. *Food Control*, *4*(2), 89−96.

Using natural antimicrobials to enhance the safety and quality of fresh and processed fruits and vegetables: types of antimicrobials

13

M.V. Alvarez[1], *M. del R. Moreira*[1], *S.I. Roura*[1], *J.F. Ayala-Zavala*[2], *G.A. González-Aguilar*[2]
[1]Universidad Nacional de Mar del Plata, Buenos Aires, Argentina; [2]Centro de Investigación en Alimentación y Desarrollo, Hermosillo, Mexico

13.1 Introduction

Fruits and vegetables are an important source of vitamins and minerals in the human diet. Moreover, the consumption of these foods is related to the prevention and alleviation of degenerative diseases such as cardiovascular disease, cancer, and aging (Allende, Tomás-Barberán, & Gil, 2006). These beneficial health effects are attributed to the presence of antioxidants in fruits and vegetables (ascorbic acid, β carotene, and polyphenols) (Martín-Diana, Rico, & Barry-Ryan, 2008).The fresh produce industry has experienced strong growth during the last two decades, driven by increases in consumption and sales in addition to the growing amount of space allocated in supermarkets for stocking of fresh produce to satisfy consumer demand. Consumers perceive fresh produce as healthy, tasty, and convenient. These features are valued primarily by busy consumers who want to maintain their health (Garrett et al., 2003). In the face of this market growth, the fresh produce industry confronts new challenges, such as ensuring the safety of products to protect consumers and developing technologies that can sustain fresh produce quality and extend its shelf life to reach more distant markets.

Considerable research has been focused recently on the development of natural food preservatives for fresh produce, all aimed to meet consumer demands for healthy and natural products. This chapter summarizes advances in research on the use of novel natural antimicrobials for safety assurance and quality preservation of fresh and processed fruits and vegetables. The chapter also discusses natural antimicrobial effectiveness; factors affecting antimicrobial activity; and secondary responses for their use in sensory acceptability and antioxidant activity in the treated product.

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13.2 Fresh and processed fruits and vegetables: advances and challenges

13.2.1 Microbiological aspects affecting quality of fruits and vegetables

Microbial growth is a factor affecting the safety and shelf life of fresh and processed fruits and vegetables. Vegetables are composed mainly of water and have a high water activity (>0.99). The intracellular pH is another important intrinsic factor in vegetables, and ranges generally from 4.9 to 6.5. These properties allow the growth of microorganisms in vegetables when nutrients become available. Plants are subjected to physical damage by different factors before, during, and after harvest by insects or rodents, handling, and postharvest processing as peeling or cutting. Due to damage on the surface, nutrients such as organic acids and sugars are released from the plant tissue, promoting microbial growth.

The dominant bacterial population on fresh and minimally processed vegetables is composed of species belonging to the *Pseudomonadaceae* (especially *Pseudomonas fluorescens*) and *Enterobacteriaceae* (especially *Erwinia herbicola* and *Rahnella aquatilis*), and some species belonging to the lactic acid bacteria (LAB) (especially *Leuconostoc mesenteroides*). (Ayala-Zavala, Del Toro-Sánchez, Alvarez-Parrilla, & González-Aguilar, 2008; Ragaert, Devlieghere, & Debevere, 2007). Yeast growth in vegetables is also important; many different yeast species have been identified, including species of *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, and *Pichia*. Molds are less important constituents of the microflora of vegetables because the slightly acid to neutral pH in the microflora favors bacteria and yeasts, which overgrow molds (Ayala-Zavala, Del Toro-Sánchez, et al., 2008; Ragaert, Jacxsens, Vandekinderen, Baert, & Devlieghere, 2010).

In the case of fruits, intrinsic properties (pH less than 4.0) favor the growth of yeasts and molds, which have a competitive advantage over bacteria to proliferate in fruit tissues, thanks to their ability to grow at lower pH range (2.2−5.0) (Corbo, Speranza, Campaniello, D'Amato, & Sinigaglia, 2010). The typical microflora found in fresh and processed fruits includes fungi (such as *Botrytis*, *Rhizopus*, *Mucor*, *Aspergillus*, *Penicillium*, *Eurotium*, and *Wallemia*), yeasts (*Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora*, *Candida*, *Debaryomyces*, *and Pichia*), and bacteria, mainly LAB (Corbo et al., 2010; Ragaert et al., 2010).

The composition of fruits and vegetables determines the type of spoilage that will be produced. With regard to sugar-rich products such as carrots and most fruits, growth of yeasts and LAB is favored, resulting in off odors caused by microbial proliferation and the production of acids such as lactic acid, acetic acid, malic acid, and others. In products with lower sugar content, such as lettuce, spoilage caused by soft rot bacteria such as *Pseudomonas* sp. commonly occurs. Thus, mainly textural and visual defects affect quality of these types of products (Ragaert et al., 2010). Different microorganisms are able to produce pectinolytic enzymes, which could influence textural changes in vegetables by degrading the middle lamella and the primary cell wall. Several species of *Erwinia* and *Pseudomonas* are the most common pectinolytic bacteria in minimally processed vegetables (Ragaert et al., 2007).

13.2.2 Postharvest losses of fruits and vegetables

Fungal plant pathogens are responsible for large postharvest losses of fruits and vegetables due to the damages they cause. Fruits are very susceptible to fungal attack because of their low pH and high content of moisture and nutrients, so they can suffer from rot or be rendered unfit for consumption due to the presence of mycotoxins produced by fungal pathogens (e.g., patulin produced by *Penicillium expansum* and ochratoxin A produced by some species of *Aspergillus* such as *Aspergillus carbonarius* and *Aspergillus niger*) (Moss, 2008). The use of fungicides is widespread throughout the world and is the main strategy to control postharvest diseases in fruits and vegetables. The use of synthetic chemicals such as fungicides has been restricted for various reasons, mainly their carcinogenic and teratogenic effects, their residual toxicity, their long-term degradation, environmental pollution, and other side effects in human health (Tripathi & Dubey, 2004). Another problem that affects the use of these chemicals is that postharvest pathogens can develop resistance to the most commonly used fungicides (Dianez, Santos, Blanco, & Tello, 2002).

Given these difficulties, the fruit and vegetable industry has faced the challenge of developing alternative strategies for control of post-harvest decay that are safe for human health and the environment, and that are well received and accepted by consumers (Tripathi & Dubey, 2004). In recent years, there has been a growing interest in the search for natural compounds, mainly of vegetable origin, that can replace synthetic antimicrobials. In this respect, various compounds or natural extracts have been studied as natural preservatives for fruits and vegetables, which will be discussed in the following section.

13.2.3 Perishability of fresh-cut produce

Fresh-cut produce, within the fresh produce market, is defined by the International Fresh-cut Produce Association as "any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form, but remains in a fresh state" (Garrett et al., 2003). As noted, consumers demand fresh, safe, nutritious, and inexpensive products and furthermore, many of them seek the opportunity to save time by buying ready-to-eat fruits and vegetables. The most important motivation for purchasing fresh-cut vegetables is convenience and speed, although health and nutritional value of these products are important attributes valued by consumers (Ragaert, Verbeke, Devlieghere, & Debevere, 2004).

Consumption of fresh-cut produce has increased rapidly in recent years, mainly in Europe and the United States, where demand is expected to represent 20% of the total food market in the near future (Siddiqui, Chakraborty, Ayala-Zavala, & Dhua, 2011). Within the wide variety of fresh-cut products in the market, the most common are the mixtures of vegetables, such as salad vegetables, but also carrots, broccoli, cauliflower, cabbage, tomatoes, and others sold individually (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). To prepare these products, raw material is subjected to operations such as washing (to remove any dirt and reduce microbial growth), peeling, cutting/slicing, and packaging (Krasaekoopt, Bhandari, Sinha, & Hui, 2011). Minimally

processed products experience rapid loss of quality due to increased respiration rate, weight loss, loss of firmness, color changes, and microbial spoilage. Operations such as peeling and cutting generate major damage to plant tissue and cause the release of intracellular contents. These may cause some unfavorable biochemical reactions such as browning, loss of texture, and off flavor, as well as nutritional and microbial quality deterioration in fresh-cut products (Allende et al., 2006). It is well known that microbial growth on these processed products is much higher than in products that have not been cut. Cell damage leads to the release of and contact between substrates and enzymes, promoting the growth of native or exogenous microorganisms. Thus, shelf life of fresh-cut fruits and vegetables is limited mainly by microbial growth and enzymatic activity (Ayala-Zavala, González-Aguilar, & Del Toro-Sánchez, 2009).

13.2.4 Safety aspects in fresh produce

The increased consumption of fresh and fresh-cut produce in the last years in the United States has resulted in an increase in the reported number of foodborne illness outbreaks associated with these products. Several factors can explain this, including consumption of meals prepared outside the home, the use of abusive temperatures in storing produce, and the transportation and distribution of fresh and processed fruits and vegetables to distant geographic locations (Harris et al., 2003). During 1998−2008, a total of 13,352 foodborne disease outbreaks, causing 271,974 illnesses, were reported in the United States. From these data, Painter et al. (2013) attributed 46% of illnesses to produce commodities (fruits, nuts, and vegetables) and estimated that this group was responsible for 38% of hospitalizations and 23% of deaths each year. In addition, authors indicated that more illnesses were associated with leafy vegetables (22%) than any other commodity, and considered this type of food the second most frequent cause of hospitalizations (14%) and the fifth most frequent cause of death (6%).

The microorganisms associated with disease outbreaks connected with the consumption of fresh and fresh-cut produce include bacteria, viruses, and parasites. The most common pathogenic bacteria in outbreaks belong to the family *Enterobacteriaceae* and are found in the intestinal tract and in the feces of humans and animals (Ayala-Zavala et al., 2009). *Escherichia coli* O157:H7, *Salmonella* spp., and *Shigella* spp. are the most frequently reported (Berger et al., 2010; Harris et al., 2003). It is important to mention another pathogen of particular concern for fresh-cut produce safety: *Listeria monocytogenes.* It has been isolated from soil, water, sewage, a large variety of vegetables, and the feces of humans and animals (Beuchat, 1996).

Fresh and processed fruits and vegetables can be contaminated with the previously mentioned pathogenic microorganisms, which cause disease by several mechanisms (Mostowy & Cossart, 2012; Obrig, 2010; Sterzenbach, Crawford, Winter, & Bäumler, 2013). Contamination of fruits and vegetables can occur during harvesting, processing, handling, transportation, as well as at commercial establishments, in food services, or at home. The pathogens enter the food directly or indirectly from soil, water, animals, insects, human handling, and contaminated surfaces (Berger et al., 2010). Attachment

of cells is necessary for colonization and subsequent transmission of pathogens via the edible parts of plants. In fact, once attached it is very difficult to remove the pathogens from contaminated plant tissues by washing. The mechanisms that bacterial pathogens use to colonize fruits and vegetables were reviewed by Berger et al. (2010).

13.2.5 The need for developing natural preservation strategies

Consumers are increasingly aware of health issues and their relationship with food. Concerned buyers have been increasingly rejecting synthetic disinfectants and additives in general. Some of these synthetic compounds are widely deployed, for example chlorine, which is of particular focus here because it has been shown to produce residual derivatives that at certain doses can cause adverse health and environmental effects (EPD, 2000; Rico et al., 2007). It is known that chlorine is involved in chemical reactions producing carcinogenic halogenated by-products (Lee, Costello, & Kang, 2004). Treatments with chlorinated water have been and are traditionally applied to decontaminate fresh produce, but in several countries (Germany, The Netherlands, Switzerland, and Belgium) the use of chlorine in fresh-cut produce is actually prohibited (Corbo et al., 2010).

Thus, naturally occurring antimicrobials have gained popularity among researchers and food producers looking for healthy alternatives to replace synthetic compounds. Natural antimicrobials can be applied as preservatives in fruits and vegetables to ensure safety, protect their quality and extend their shelf life. These agents can be obtained from animal, plant, and microbial sources, where they play a role in the natural defense system of their hosts. Essential oils, flavor compounds, phenolic compounds, chitosan, isothiocyanates, LAB, and bacteriocins have been applied to fresh and processed fruits and vegetables. Their effectiveness as antimicrobials will be discussed in the following section.

13.3 Natural antimicrobials used in assuring the safety and quality of fresh and processed fruits and vegetables: antimicrobials from plant essential oils

Several antimicrobial compounds or extracts, derived from different sources, have been studied in the literature regarding their potential to combat pathogenic and spoilage microorganisms. Some of them have been effectively applied to fresh and processed fruits and vegetables to prevent decay and assure safety (Table 13.1). Advances in the use of natural antimicrobials in fresh and processed fruits and vegetables are described in this section.

Plants, herbs, and spices have been found to be rich sources of natural antimicrobials, many of which are phenolic compounds, terpenoids, aldehydes, esters, or sulfur-containing compounds. These agents can be found in leaves, seeds, flowers, bulbs, and roots in nature. These compounds are secondary metabolites produced by plants as a defense mechanism against phytopathogens and insect attack, but many

Table 13.1 Effect of natural antimicrobials on the decay and safety status of fresh and processed produce

Group	Antimicrobial agent	Treated produce	Antimicrobial effect	References
Essential oils and components	Thyme essential oil	Romaine lettuce, carrots	*Escherichia coli* O157:H7	Singh, Singh, Bhunia, and Stroshine (2002)
	Basil methyl chavicol	Lettuce, iceberg green	Native microflora	Wan, Wilcock, and Coventry (1998)
	Oregano essential oil	Eggplant salad	*E. coli* O157:H7	Skandamis and Nychas (2000)
	Cinnamaldehyde, thymol	Alfalfa seeds	*Salmonella* spp.	Weissinger et al. (2001)
	Carvacrol, cinnamic acid	Kiwifruit, honeydew melon	Native microflora	Roller and Seedhar (2002)
	Cassia essential oil	Cherry tomatoes	*Alternaria alternata*	Feng and Zheng (2007)
	Oregano, savory, and thyme essential oils	Apple	*Botrytis cinerea* and *Penicillium expansum*	Lopez-Reyes et al. (2010)
	Tea tree essential oil	Butterhead lettuce (preharvest application)	Native microflora	Goñi et al. (2013)
	Garlic essential oil	Fresh-cut tomatoes	Mesophilic bacteria, yeast, and molds	Ayala-Zavala and González-Aguilar (2010)
	Cinnamon leaf essential oil	Fresh-cut peaches	Native microflora	Ayala-Zavala et al. (2013)
	Clove and tea tree essential oils	Romaine lettuce	Native microflora	Ponce, Roura, and Moreira (2011)
	Eucalyptus, clove, and tea tree essential oils	Swiss chard	Native microflora	Ponce, Del Valle, and Roura (2004a)

	Oregano and rosemary essential oils in combination	Iceberg lettuce leaves, beet, and rocket	Native microflora, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophila*	De Azeredo et al. (2011)
	Oregano and rosemary essential oils in combination	Table grapes	*Aspergillus flavus* and *Aspergillus niger*	De Sousa et al. (2013)
Aldehydes	Hexanal	Fresh-cut apple	Native microflora	Lanciotti, Corbo, Gardini, Sinigaglia, and Guerzoni (1999)
	Hexanal, hexyl acetate and 2-(E)-hexenal	Fresh-cut apple	*E. coli* and *Salmonella enteritidis* and *L. monocytogenes*	Lanciotti et al. (2003)
	Hexanal and *trans*-2-hexenal	Fresh-cut apple	*P. subpelliculosa*	Corbo et al. (2000)
	Vanillin (4-hydroxy-3-methoxybenzaldehyde)	Fresh-cut apple	Native microflora, *L. innocua*	Rojas-Graü et al. (2007); Rupasinghe, Boulter-Bitzer, Ahn, and Odumeru (2006)
	Vanillin (4-hydroxy-3-methoxybenzaldehyde)	Fresh-cut mangoes	Native microflora	Ngarmsak et al. (2006)
Esters	Methyl jasmonate	Fresh-cut pineapple	Native microflora	Martínez-Ferrer and Harper (2005)
	Methyl jasmonate	Fresh-cut kiwifruit	Native molds	Wang and Buta (2003)
	Methyl jasmonate	Strawberry	Molds	Ayala-Zavala et al. (2005)
	Methyl jasmonate	Fresh-cut tomato	Native microflora	Ayala-Zavala, Del Toro-Sánchez, et al. (2008)

Continued

Table 13.1 Continued

Group	Antimicrobial agent	Treated produce	Antimicrobial effect	References
Polyphenols	Grape seed extract	Fresh-cut cucumber and lettuce	*L. monocytogenes* and *Salmonella* sp.	Xu et al. (2007)
	Pomegranate peel extract	Minimally processed broccoli	Mesophilic and psychrotrophic bacteria	Alvarez, Ponce, and Moreira (2013)
	Resveratrol	Tomatoes, grapes, avocado, green and red peppers	Yeast and molds	Jiménez et al. (2005)
	Clove extract	Lettuce	*Salmonella* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes*	Kim et al. (2011)
Isothiocyanates	Allyl isothiocyanate	Pears	*P. expansum*	Mari et al. (2002)
	Allyl and butenyl isothiocyanate	Nectarines and peaches	*M. laxa*	Mari et al. (2008)
	Allyl and ethyl isothiocyanate	Apples	*P. expansum* and *B. cinerea*	Wu et al. (2011)

Polysaccharides	Chitosan	Lettuce seeds	Native microflora	Goñi, Moreira, Viacava, and Roura (2013a)
	Chitosan	Fresh-cut papaya	Native microflora	González-Aguilar et al. (2009)
	Chitosan	Minimally processed broccoli	Mesophilic and psychrotrophic bacteria, *E. coli* O157:H7, and *L. monocytogenes*	Alvarez et al. (2013)
Lactic acid bacteria (LAB)	*L. mesenteroides*, *L. lactis*, and *Weissella* spp. strains	Golden delicious apple and iceberg lettuce	*S.* Typhimurium, *E. coli* and *L. monocytogenes*	Trias et al. (2008)
	Lactobacillus rhamnosus GG	Fresh-cut apple	*L. monocytogenes*	Alegre et al. (2011)
Bacteriocins	Nisin combined with EDTA, sodium lactate and potassium sorbate	Whole and fresh-cut cantaloupe	Mesophilic and LAB, *Salmonella*	Ukuku and Fett (2002); Ukuku and Fett (2004)
	Nisin and coagulin	Fresh-cut lettuce	*L. monocytogenes*	Allende et al. (2007)

of them have demonstrated inhibition or inactivation of bacterial and fungal species (Davidson, Critzer, & Taylor, 2013).

Given the growing interest in making healthier, safe, and natural food, the use of essential oils obtained from plants as food additives is a good alternative. Spices and their essential oils show varying degrees of antimicrobial activity. A large variety of plant and spice-based antimicrobials are used in food products, including fresh and processed fruits and vegetables, to extend their shelf life, reduce or eliminate pathogenic bacteria, and to improve the overall quality (Ayala-Zavala et al., 2009; Burt, 2004; Moreira, Ponce, Del Valle, & Roura, 2007; Ponce, DelValle, & Roura, 2004b; Tzortzakis, 2009). Essential oils or volatile oils are aromatic oily liquids that can be obtained by various methods from plant material (flowers, leaves, seeds, buds, twigs, bark, herbs, wood, fruits, and roots) (Burt, 2004). There are several methods for extracting essential oils. Low- or high-pressure distillation, employing boiling water or hot steam, is the most commonly used method for commercial production of essential oils. However, other extraction techniques, such as the use of microwaves or liquid carbon dioxide, can be used (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Bayramoglu, Sahin, & Sumnu, 2008; Eikani, Golmohammad, & Rowshanzamir, 2007; Guan, Li, Yan, Tang, & Quan, 2007).

The greatest antimicrobial activity was reported for oregano, cinnamon, clove, and thyme essential oils and their main components carvacrol, cinnamaldehyde, eugenol, and thymol, respectively (Davidson et al., 2013). Other essential oils or extracts from melissa, eucalyptus, basil, bay, citrus, lemongrass, rosemary, savory, and tea tree have demonstrated moderate to high activity in some studies against selected microorganisms (Burt, 2004; Moreira, Ponce, del Valle, & Roura, 2005). The composition of essential oils is variable and depends largely on the geographical area and the time of harvest. Some plants can be composed of up to 85% essential oil; others have oil as only trace components (Bauer, Garbe, & Surburg, 2001). It has been shown that minor components play a critical role in the antimicrobial properties of the essential oil, possibly showing synergistic effects along with other compounds (Chang et al., 2008). Given the various groups of chemical compounds present in essential oils, their antibacterial activity is not attributable to a single mechanism but to a combination of various forms of action.

The hydrophobic nature of essential oil components allows them to embed in the lipids of the bacterial cell membrane, causing damage in structures and increasing permeability. As a consequence, leakage of ions and other cell contents occurs. Also, depletion of proton motive force, coagulation of cytoplasm, inactivation of essential enzymes, and disturbance of genetic material functionality can affect cell viability (Ayala-Zavala et al., 2009; Burt, 2004).

A wide variety of essential oils have proven to be powerful antimicrobials; however, their aromatic volatile constituents can be absorbed and greatly affect the sensory characteristics of the food. As the aroma of fruits and vegetables is a key marketing feature of these foods, it is necessary to consider the sensory impact generated by the application of essential oils as biopreservatives in fruits and vegetables (Ayala-Zavala, Oms-Oliu, et al., 2008). To make a natural agent effective as an antimicrobial through the shelf life of a plant product, generally it has to be applied in high

concentrations. However, if this antimicrobial negatively affects the taste or smell of the food product, potential consumers will not accept it. Thus, the challenge is to achieve a correct or consistent use of antimicrobials, such as essential oils, so that they do not degrade the sensory attractiveness in plant products while they work to extend shelf life and ensure safety (Ayala-Zavala et al., 2009).

An opportunity currently lies in studying the antimicrobial properties of essential oils when they are applied during vegetable production as a preharvest sanitizing treatment. Preharvest application of essential oils is a viable alternative for the control of potential microbiological hazards in fresh produce. Goñi, Tomadoni, Moreira, and Roura (2013) applied solutions of tea tree essential oil (0.27/100 ml) and clove essential oil (0.15/100 ml) on late development stages of butterhead lettuce (14, 10, 7, 3, and 0 days before harvest). These authors demonstrated the efficacy of tea tree oil to reduce mesophilic and psychrotrophic bacteria, yeast and molds, and total coliform counts at harvest and after 5 days of refrigerated postharvest storage. The application of clove, however, was not effective in reducing postharvest microbial counts. The sensorial quality of lettuce was assessed by a panel of trained judges and authors, who reported that the overall visual quality, leaf color, texture, brightness, and browning of the lettuce were not affected by essential oil treatment. Essential oil application in preharvest stages (when lettuce plants are still at the greenhouse) may help to reduce the negative effect they have on the quality attributes when applied at postharvest, as was reported by Ponce et al. (2004b). The use of essential oils as natural preharvest sanitizers on fruits and vegetables requires more research to find novel treatments to ensure safety, preserve quality, and even extend shelf life of the products.

Many studies have focused on the use of essential oils as antifungal and antibacterial agents applied at postharvest of fruits and vegetables. The use of these extracts is an interesting alternative for postharvest decay control of fruits and vegetables caused by fungi. *In vitro* antifungal activity of several essential oils against postharvest fungal pathogens has been reported (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Tzortzakis, 2009; Tzortzakis & Economakis, 2007). The effectiveness as *in vivo* antifungals has been studied only for some essential oils and their components. Feng and Zheng (2007) reported that cassia (*Cinnamomum cassia*) essential oil could reduce postharvest diseases on cherry tomatoes caused by *Alternaria alternata*. The percentage of decayed cherry tomatoes treated with cassia oil (500 ppm) was reduced by 34% when tomatoes were artificially inoculated with the fungal pathogen. Similar results were obtained on naturally infected tomatoes. Also, several essential oils were tested to control grey mold of grapes caused by *Botrytis cinerea* during storage. The storage life of grapes treated with *Prunus persica*, *Ocumum sanctum*, and *Zingiber officinale* essential oils was found to be enhanced up to 4, 5, and 6 days, respectively (Tripathi, Dubey, & Shukla, 2008).

Furthermore, essential oils of oregano (*Origanum vulgare*), savory (*Satureja montana*), and thyme (*Thymus vulgaris)* showed efficacy on postharvest control of rot caused by *B. cinerea* and *P. expansum* on four cultivars of apples. Oils were applied as emulsions at 1% and 10% on apples artificially inoculated with pathogens (Lopez-Reyes, Spadaro, Gullino, & Garibaldi, 2010). Thymol, the main component of thyme essential oil, was used to fumigate sweet cherries, and results indicated its efficacy in

controlling postharvest grey mold rot caused by *B. cinerea* (Chu, Liu, Zhou, & Tsao, 1999) and brown rot caused by *Monilinia fructicola* (Chu, Liu, & Zhou, 2001).

In addition, the antibacterial activity of essential oils has been demonstrated by several *in vitro* studies against *L. monocytogenes*, *Salmonella* Typhimurium, *E. coli* O157:H7, *Shigella dysenteriae*, *Bacillus cereus*, and *Staphylococcus aureus* at levels between 0.2 and 10 μl/ml (Burt, 2004). Some of these essential oils and their main components have been tested by applying different methods on fresh and processed fruits and vegetables with the aim of reducing natural flora growth or controlling pathogen survival.

Cinnamaldehyde and thymol were effective against six *Salmonella* serotypes when applied in hot air (50 °C) to fumigate alfalfa seeds (Weissinger, McWatters, & Beuchat, 2001). Also, oregano essential oil, at 7−21 μl/g, inhibited the growth of *E. coli* O157:H7 and reduced final populations when applied to eggplant salad (Skandamis & Nychas, 2000). Clove, tea tree, and eucalyptus solutions (0.5−1.3 μl/ml) sprayed on organic Swiss chard leaves significantly reduced microbial counts along with refrigerated storage (Ponce et al., 2004a). Furthermore, clove and tea tree oil, applied at 2.5 and 5 μl/ml, were assayed as natural antimicrobials to control postharvest decay of romaine lettuce leaves. Three application technologies were tested: essential oils embedded in lactose capsules, by spray, and immersion in aqueous solutions. The form of application was a decisive factor in determining the effectiveness of the essential oils and the impact on sensorial quality. The use of both essential oils embedded in capsules exerted the highest inhibitory effect on the native microflora during refrigerated storage. However, immersion and capsule methods caused alterations in sensorial parameters such as color (Ponce et al., 2011).

As mentioned, when essential oils are applied to food matrices, the amounts required to significantly inhibit microbial growth often cause deleterious effects in the sensory quality of the product (flavor, odor, and color), rendering them organoleptically unacceptable (De Azeredo et al., 2011; Gutierrez, Bourke, Lonchamp, & Barry-Ryan, 2009; Ponce et al., 2011). However, the addition of subinhibitory doses of essential oils (or their components) in combination could be used to inhibit microbial growth while maintaining good sensory quality of processed food. Using more than one essential oil can enhance their individual antimicrobial capacity, thus obtaining beneficial synergies (Dimitrijević, Mihajlovski, Antonović, Milanović-Stevanović, & Mijin, 2007). De Azeredo et al. (2011) discovered this synergistic effect *in vitro* in the combined application of oregano and rosemary essential oils against *L. monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*. The synergistic combinations found *in vitro* were applied in *in vivo* on a mixture of iceberg lettuce leaves, beet, and rocket (by immersion for 5 min at 28 °C). Mixtures of both essential oils significantly reduced naturally occurring microflora counts in leaves (1.5−2.4 $\log_{10}$ reductions) and reduced the initial inocula of all tested bacteria from approximately 8−5 log CFU/g after 5 min of exposure in the artificially inoculated leaves. Sensory evaluation of vegetables sanitized with the oils revealed that these treatments did not affect their acceptability. Furthermore, De Sousa et al. (2012) followed the same methodology and found a synergistic interaction between carvacrol and 1,8 cineole (main components of oregano and rosemary essential oils, respectively) against

L. monocytogenes, *A. hydrophila*, and *P. fluorescens*. Then, synergistic combinations were applied on a mix of lettuce, chard, and rocket by immersion; the test yielded results that were similar to the previous findings.

13.4 Antimicrobials from plants: aldehydes and methyl jasmonate

13.4.1 Aldehydes

There are many naturally occurring compounds in fruits and vegetables that give them their characteristic flavors, and these compounds can also exert antimicrobial protection. Thus, they can be extracted and applied to other plant products for preservation (Tripathi & Dubey, 2004). Some of these volatile compounds are aldehydes produced through the lipoxygenase pathway to exert a key role in the defense system of plants against pathogens attack. Moreover, plant volatiles have been widely used as food flavoring agents, and most of them are generally recognized as safe (Lanciotti et al., 2004). Some components that give many fruits and vegetables their aromas, such as hexanal, 2-(E)-hexenal, *trans*-2-hexenal, and hexyl acetate, have demonstrated antimicrobial activity against spoilage and pathogenic species (Corbo, Lanciotti, Gardini, Sinigaglia, & Guerzoni, 2000; Lanciotti et al., 2003; Utama, Wills, Ben-yehoshua, & Kuek, 2002). Lanciotti et al. (2003) reported that treatments with hexanal (150 μl/l), hexyl acetate (150 μl/l), and 2-(E)-hexenal (20 μl/l) on artificially inoculated apple slices produced significant extensions in lag phase of *E. coli* and *Salmonella enteritidis* when stored at 20 °C. These treatments were more effective against *L. monocytogenes*, causing bactericidal effects (4−5 $\log_{10}$ CFU/g reduction) after 4 days of storage. These authors hypothesized that the higher resistance of Gram-negative bacteria to these natural compounds is due to the presence of the outer membrane, which can act as a barrier to hydrophobic substances.

Furthermore, Corbo et al. (2000) studied the effect of applying hexanal (0.3 μl/l, in vapor form) on sliced apple inoculated with the spoilage yeast *Pichia subpelliculosa* and packed under modified atmosphere. These authors reported a significant extension on lag phase of 13 days (at 25 °C) and 10 days (at 5 °C) when compared to control. Additionally, when *trans*-2-hexenal (0.06 μl/l) was applied in the same form as hexanal, its use extended significantly the lag phase of *P. subpelliculosa* also when stored at abusive temperatures. The antimicrobial activity of these volatile compounds was influenced by the storage temperature, which affects the vapor pressure of the used compounds.

13.4.2 Methyl jasmonate

Methyl jasmonate is a methyl ester widely distributed in the plant kingdom. It was first detected as a fragrant compound present in *Jasminum* essential oil and other plant species. Methyl jasmonate is synthesized via the lipoxygenase pathway and is known to regulate plant growth and response to environmental stresses (González-Aguilar,

Tiznado-Hernández, & Wang, 2006). This natural compound, either as a vapor or as an emulsion, has been shown to control microbial proliferation in fresh and processed fruits and vegetables (strawberries, grapefruit, pineapple, tomato, peppers, and celery) (Ayala-Zavala, Wang, Wang, & González-Aguilar, 2005; Ayala-Zavala, Oms-Oliu, et al., 2008; Martínez-Ferrer & Harper, 2005).

Ayala-Zavala, Oms-Oliu, et al. (2008) studied the effectiveness of several natural volatile compounds, including methyl jasmonate to preserve quality of fresh-cut tomato stored at 5 °C for 15 days. Natural compounds were spotted onto filter paper strips placed inside the containers. These authors demonstrated that methyl jasmonate applied at 22.4 μl/l as vapor significantly inhibited the growth of aerobic mesophilic microorganisms, coliforms, and molds in fresh-cut tomato. The authors reported that treatment with methyl jasmonate (22.4 μl/l) combined with ethanol (300 μl/l) was more effective in suppressing microbial proliferation than the individual treatments during the storage period. Furthermore, Wang and Buta (2003) studied the effect of methyl jasmonate applied at 11.2 and 22.4 μl/l as vapor on the microbial quality of fresh-cut kiwifruit. Treatments were effective in inhibiting mold proliferation during 3 weeks of storage at 10 °C.

13.5 Antimicrobials from plants: phenolic compounds and isothiocyanates

13.5.1 Phenolic compounds

Polyphenols are secondary metabolites of higher plants that play a key role in their defense against plant pathogens and microorganisms, and as a response to abiotic stress conditions such as rain or ultraviolet radiation (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). This group is composed of a variety of molecules with polyphenolic structure, and according to their chemical structure they are classified as phenolic acids, flavonoids, stilbenes, and lignans (Blasa, Gennari, Angelino, & Ninfali, 2010). The antimicrobial activity of polyphenols found in fruit, vegetables, and medicinal plants has been extensively investigated against a wide range of microorganisms. The most effective polyphenolic compounds are the flava-3-ols (e.g., catechin), flavonols (e.g., quercetin), and tannins, given their broad spectrum of action and their high antimicrobial power. Furthermore, it has been shown that many of them have the ability to suppress virulence factors, such as biofilm formation inhibition and the neutralization of bacterial toxins (Daglia, 2012).

Some classes of polyphenols have been proposed as alternatives for the development of new food preservatives because of their effectiveness as antimicrobials and because they are natural and safe. In fact, some studies have tested plant extracts rich in polyphenols to ensure safety, extend shelf life, and reduce spoilage of fresh and processed fruits and vegetables (Alvarez et al., 2013; Ayala-Zavala, Rosas-Domínguez, Vega-Vega, & González-Aguilar, 2010; Cruz-Valenzuela, Carrazco-Lugo, Vega-Vega, Gonzalez-Aguilar, & Ayala-Zavala, 2013; Jiménez et al., 2005; Kim

et al., 2011; Martín Diana et al., 2008; Vega−Vega et al., 2013; Xu et al., 2007). The processing of fruits and vegetables usually generates a great amount (up to 30%) of by-products and inedible parts (Ajila, Aalami, Leelavathi, & Prasada-Rao, 2010). These parts, which are normally discarded, include skins, peels, and seeds rich in polyphenols that may be very potent as antioxidants and antimicrobials. Some studies suggest using these by-products of fruits and vegetables as effective antimicrobials (Ayala-Zavala et al., 2010).

Wine processing generates valuable by-products from the point of view of their biological properties, such as grape seed extract from *Vitis vinifera.* This substance is extracted, dried, and purified to produce a compound-rich polyphenolic extract. It has been shown that the grape seed extract presents antimicrobial activity due to its high polyphenol content, primarily catechin, epicatechin, and epicatechin-3-O-gallate (Perumalla & Hettiarachchy, 2011). Xu et al. (2007) applied grape seed extract to reduce the survival and growth of pathogenic bacteria including *L. monocytogenes* and *Salmonella* spp., in whole and fresh-cut cucumber and lettuce. These authors showed that the use of grape seed extract in a sanitizing solution (0.1% w/v) significantly reduced the populations of both pathogens after submerging inoculated vegetables (2.5−2.8 $\log_{10}$ reductions compared with water treatment). Additionally, grape seed extract inhibited the growth of total aerobes in uninoculated cucumber (stored for 8 days at 10 °C) and lettuce (21 days at 4 °C) and preserved sensory quality of treated vegetables.

Pomegranate (*Punica granatum* L.) is a fruit widely studied for its biological activity. The antimicrobial activity of pomegranate peel extracts has been demonstrated against pathogenic bacteria *(E. coli*, *L. monocytogenes*, *S. aureus*, and *Y. enterocolitica*, among others) (Al-Zoreky, 2009; Alvarez, Moreira, & Ponce, 2012); this activity is related to the high content of polyphenols (flavonoids and tannins) present in pomegranate peels. In addition, the application of pomegranate peel extract—rich in ellagic acid and punicalagins—to reduce spoilage of minimally processed broccoli has been tested. The application of this extract at 120 μg/ml reduced the development of psychrotrophic and mesophilic bacteria (2 $\log_{10}$ reduction) during refrigerated storage (Alvarez et al., 2013).

Resveratrol (3,5,4′-trihydroxystilbene) has been described as one of the most important molecules for resistance to fungal diseases of the grapevine. It has been shown that this compound has a broad activity spectrum as an antifungal, and has been highly valued for its effectiveness against the phytopathogenic fungus *B. cinerea*, which causes significant losses for vineyard owners worldwide (Filip et al., 2003). For this reason, resveratrol is considered a natural alternative to the use of synthetic fungicides. Resveratrol-rich extracts can be obtained from grape skins, berries, nuts, and medicinal plants as *Polygonum cupsidatum* (Pirola & Fröjdö, 2008). Jiménez et al. (2005) demonstrated its antifungal power, applying aqueous solutions of this compound (0.16 mM) on tomatoes, grapes, apples, avocado, and green and red peppers. This treatment reduced postharvest decay in all fruits, and it was possible to improve fruit shelf life, not only inhibiting yeast and molds growth but also reducing water losses, without affecting nutritional content.

Several other studies have been conducted in different countries to prove antimicrobial efficacy of plant extracts, mainly those rich in polyphenols (Ahmad & Beg, 2001; Kim et al., 2011; Rabanal, Arias, Prado, Hernández-Pérez, & Sánchez-Mateo, 2002; Rauha et al., 2000). Plant extracts can be prepared from roots, leaves, seeds, stems, and fruits using different solvents such as ethanol, methanol, acetone, and water. Kim et al. (2011) evaluated the antimicrobial activity of 12 ethanolic plant extracts against pathogens such as *Salmonella* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes. Syzygium aromaticum* (clove), rich in eugenol, showed the highest *in vitro* inhibitory effect determined by disk diffusion assay. Also, clove extract was tested in different concentrations (1, 5, and 10% v/v) on lettuce previously inoculated with the same pathogens. Treatments were carried out by dipping the leaves for 1, 3, 5, and 10 min. As a result, clove extract applied at 5% significantly reduced microbial levels of *S.* Typhimurium and *E. coli* O157:H7 after 3 min, and more than 2 $\log_{10}$ reductions were observed for 10 min treatment. In case of 10% extract, significant reduction occurred in just 1 min, and more than 3 $\log_{10}$ reductions were observed after 3 min treatment.

13.5.2 Isothiocyanates

Isothiocyanates consist of aliphatic and aromatic compounds resulting from the reaction between glucosinolates and the endogenous enzyme myrosinase in cruciferous vegetables (cauliflower, broccoli, and cabbage) that occur when tissues are damaged (Wilson et al., 2013). It has been reported that these compounds exert inhibitory activity against several fungi and pathogenic and food spoilage bacteria including species from *Escherichia*, *Klebsiella*, *Salmonella*, *Bacillus*, *Serratia*, *Staphylococcus*, and *Listeria* (Davidson et al., 2013; Mari, Leoni, Bernardi, Neri, & Palmieri, 2008; Wilson et al., 2013). Gram-negative bacteria generally are more sensitive to this group of compounds than Gram-positive bacteria. It has been hypothesized that the mechanism of antimicrobial action of isothiocyanates is related to the interaction of these compounds with sulfhydryl-containing enzymes that are involved in vital functions of the cell and damage cellular structures (Luciano & Holley, 2009).

Given the volatile nature of many isothiocyanates, these compounds may be used as preservatives in fruits and vegetables, applied as vapors in storage operations or in modified atmosphere packaging (Mari, Leoni, Iori, & Cembali, 2002). Mari et al. (2008) demonstrated that natural allyl and butenyl isothiocyanate vapors were effective in reducing brown rot (caused by *Monilinia laxa)* in artificially inoculated nectarines and peaches. Infected fruits were exposed for 3−6 h in an isothiocyanate-enriched atmosphere and then incubated for at least 3 days at 20 °C. Also, an allyl isothiocyanate-enriched atmosphere was used to treat pears stored 24 h at room temperature. This treatment was effective to control the growth of *P. expansum* (Mari et al., 2002). Wu, Zhang, Zhang, Zeng, and Lin (2011) investigated synergistic interactions between isothiocyanates as antifungals with the aim of reducing required concentrations. Allyl isothiocyanate and ethyl isothiocyanate were applied singly and in combination (as vapors) to control fungi on apples artificially infected with *P. expansum* and *B. cinerea.* These authors reported that use of single

isothiocyanates required higher concentrations (approximately twofold) than did the combinations of allyl isothiocyanate and ethyl isothiocyanate when supplied at the ratio of 3:1 to achieve the same level of control of *P. expansum* and *B. cinerea* spore germination and mycelial growth.

These studies demonstrate the potential use of biofumigation based on isothiocyanates to control postharvest fungal diseases of fruits and vegetables. However, further studies are necessary to evaluate quality and sensorial changes in products caused by these treatments.

13.6 Chitosan is not from plant origin

Chitosan, of animal origin, is the most studied antimicrobial agent for preserving quality and assuring safety of fresh and processed fruits and vegetables. It is the second most abundant biopolymer on earth after cellulose; it is a high molecular-weight cationic polysaccharide composed of β-1,4-linked 2-acetamido-2-deoxy-glucopyranose, and 2 amino-2-deoxy-glucopyranose, which exhibits antibacterial and antifungal activity as well as film-forming properties. It is obtained commercially by deacetylation of chitin, which is an abundant constituent in crustaceans, insects, and fungi (Sebti, Martial-Gros, Carnet-Pantiez, Grelier, & Coma, 2005).

Chitosan has shown a wide inhibition spectrum for bacteria (Gram-positive and Gram-negative), and also for yeast and molds (Liu et al., 2006). Effectiveness of chitosan as an antimicrobial varies depending on the molecular weight of the polymer, degree of acetylation, pH, temperature, and interfering compounds in foods such as proteins and lipids (Davidson et al., 2013). Antimicrobial activity of chitosan is generally associated with its positive charge of the amino group (NH_2), which can create a polycationic structure and interact with anionic components such as lipopolysaccharides and proteins of the membrane cell surface, leading to the leakage of intracellular constituents (Liu et al., 2006).

The antimicrobial properties of chitosan-based coatings applied to fresh and processed fruits and vegetables (and also to seeds) have been evaluated by several researchers to study the preservation of microbiological quality. Goñi et al. (2013) evaluated the efficiency of chitosan as a decontamination alternative for lettuce seed. Chitosan solutions (10 g/l) significantly reduced naturally occurring mesophilic bacteria, coliforms, yeast, and molds on seeds after 10 min of exposure, without affecting germination percentage. Furthermore, González-Aguilar et al. (2009) evaluated microbiological quality of fresh-cut papaya cubes treated with chitosan coatings of low, medium, and high molecular weights. Treatments were able to reduce the growth of mesophilic bacteria, molds, and yeast compared to controls during refrigerated storage. Medium molecular weight chitosan coating, applied at 2% w/v, showed the highest antimicrobial activity. Chitosan coating efficiency was also evaluated on minimally processed broccoli by Alvarez et al. (2013). This coating, applied by immersion at 10 g/l, produced a significant reduction in mesophilic and psychrotrophic bacteria counts (2.5−3.5 $\log_{10}$ CFU/g) compared to control samples, between days two and seven of refrigerated storage. Also, it was effective as an inhibitor of the growth

of *E. coli* and *L. monocytogenes* artificially inoculated on broccoli florets, showing a bacteriostatic effect during the storage and a 1.5 to 2 $\log_{10}$-reduction on pathogen counts compared to control samples.

13.7 Natural antimicrobials of microbial origin: lactic acid bacteria (LAB) and bacteriocins

13.7.1 Lactic acid bacteria

It is well known that many LAB act as probiotics, offering health benefits for consumers. However, these bacteria can also play a protective role against pathogenic microorganisms found in food, acting as biopreservatives. When applied to a product during storage, they compete with pathogens for nutrients and produce antimicrobial agents like organic acids and bacteriocins (antimicrobial peptides) (Rydlo, Miltz, & Mor, 2006). Some authors have studied the application of microbial cultures with the ability to control the development of pathogens and spoilage microorganisms as a natural strategy to extend the life and ensure the safety of vegetable products. Trias, Bañeras, Badosa, and Montesinos (2008) studied the biopreservative potential of several lactic acid bacterial strains isolated from fresh fruits and vegetables (*Leuconostoc mesenteroides, Lactococcus lactis*, and *Weissella* spp.). Strains were tested in wounded golden delicious apples and iceberg lettuce leaf cuts artificially inoculated with foodborne pathogens. The treatment of both products with antagonistic strains reduced the cell count of *S.* Typhimurium and *E. coli* by 1−2 $\log_{10}$ CFU/g, whereas the growth of *L. monocytogenes* was completely inhibited. These results showed that LAB are more efficient for controlling Gram-positive bacteria than Gram-negative bacteria.

Furthermore, Alegre, Viñas, Usall, Anguera, and Abadias (2011) studied the effectiveness of the probiotic *Lactobacillus rhamnosus* GG as an antagonistic bacterium. These authors applied the protective culture (10^8 CFU/ml) on fresh-cut apples inoculated with *Salmonella* and *L. monocytogenes*, and results showed that *Salmonella* was not affected by the presence of *L. rhamnosus* GG but L. *monocytogenes* population was 1-$\log_{10}$ unit lower than untreated controls during storage. The researchers demonstrated that the quality of apples was not affected by the addition of the probiotic and that *L. rhamnosus* GG counts were kept over the generally recommended level for probiotic action (10^6 CFU/g) (Kailasapathy & Chin, 2000) for the first 14 days of storage.

13.7.2 Bacteriocins

The main antimicrobial compounds of microbial origin are bacteriocins, produced by various Gram-positive bacteria, including LAB, during their growth. These polypeptides, of low molecular weight, give the producing microorganism a competitive advantage over other similar microorganisms. A large number of bacteriocins are produced by Gram-positive bacteria and are classified according to their chemical structure and action mechanism as antimicrobials.

Nisin is one of the most studied bacteriocins for its application in foods. It is a lantibiotic small peptide (34 amino acids) produced by many strains of *Lactococcus lactis.* This bacteriocin shows antimicrobial action only against Gram-positive bacteria, including *Bacillus cereus*, *Clostridium botulinum*, *Clostridium sporogenes*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *L. monocytogenes*, *Micrococcus*, *Pediococcus*, *Staphylococcus* and others, and generally it does not inhibit Gram-negative bacteria, yeasts, or molds (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martín-Belloso, 2009). Nisin has been approved by the United States Food and Drug Administration for use as a food preservative in certain applications. It is hypothesized that the primary action site of nisin is the cytoplasmic membrane of the cells, where it forms pores affecting membrane integrity. This membrane damage results in loss of intracellular contents and depletion of proton motive force (Davidson & Zivanovic, 2003).

Nisin and other bacteriocins have been studied to reduce the risk of *L. monocytogenes* on fresh-cut iceberg lettuce. Allende et al. (2007) demonstrated the efficacy of nisin and coagulin solutions to inhibit the growth of *L. monocytogenes* in *in vitro* assays. Also, the effect of bacteriocins (applied by washing) on the survival and growth of *L. monocytogenes* was evaluated in fresh-cut lettuce during refrigerated storage. Immediately after the application of solutions, a bactericidal effect against the pathogen was observed (1.2−1.6 $\log_{10}$ reduction) but, during storage, the inhibitory effect was lower, exerting minimal control over the proliferation of *L. monocytogenes.*

The effectiveness of nisin as a biopreservative in fruits has been tested in combination with other antimicrobial treatments. Ukuku and Fett (2004) demonstrated the efficacy of nisin solution (50 μg/ml) combined with ethylenediaminetetraacetic acid (EDTA) (0.02 M), sodium lactate (2% v/v), and potassium sorbate (0.02% v/v) to reduce *Salmonella* counts on fresh-cut cantaloupe artificially inoculated with pathogens. Also, in another study, nisin solution (10 μg/ml) combined with EDTA (0.02 M) reduced mesophilic aerobic and LAB counts (2 $\log_{10}$ CFU/g) on fresh-cut cantaloupe after washing it with the combined solution. However, during refrigerated storage, this treatment was not effective to reduce the growth of *Pseudomonas*, yeast, and molds (Ukuku & Fett, 2002).

13.8 Conclusion and future trends

Preservation and safety of fresh and processed fruits and vegetables are topics that concern authorities, food producers, and scientists due to the growing consumer demand for these foods. Given the limitations in the use of synthetic disinfectants, such as chlorine, it is important to continue the search for natural and safe preservation alternatives. In the last two decades, significant progress has been made, but further research is necessary to develop new technologies such as the use of natural preservatives (plant extracts, bacteriocins and others), optimizing their application methods to reach higher efficiency. The use of natural antimicrobials on fruits and vegetables without negatively affecting the sensory properties is still a challenge for researchers. To inhibit

proliferation or eliminate pathogenic bacteria, the required concentrations of these agents are very high, and at such concentrations they can affect the sensory qualities of foods. Hence, combining these treatments with physical barriers, such as ultrasound and high pressures, may be a viable strategy to ensure the safety, nutritional, and sensory qualities of fresh produce. Furthermore, not enough research has been done on the potential synergistic combinations of natural agents to reduce microbial growth in fruits and vegetables; using more than one natural agent can reduce the concentrations needed, thereby minimizing the effect on organoleptic characteristics of the treated food.

References

Ahmad, I., & Beg, A. Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of Ethnopharmacology*, *74*, 113−123. http://dx.doi.org/10.1016/S0378-8741(00)00335-4.

Ajila, C. M., Aalami, M., Leelavathi, K., & Prasada-Rao, U. J. S. (2010). Mango peel powder: a potential source of antioxidant and dietary fiber in macaroni preparations. *Innovative Food Science & Emerging Technologies*, *11*, 219−224. http://dx.doi.org/10.1016/j.ifset.2009.10.004.

Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *International Journal of Food Microbiology*, *134*, 244−248. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.07.002.

Alegre, I., Viñas, I., Usall, J., Anguera, M., & Abadias, M. (2011). Microbiological and physicochemical quality of fresh-cut apple enriched with the probiotic strain *Lactobacillus rhamnosus* GG. *Food Microbiology*, *28*, 59−66. http://dx.doi.org/10.1016/j.fm.2010.08.006.

Allende, A., Martínez, B., Selma, V., Gil, M. I., Suárez, J. E., & Rodríguez, A. (2007). Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenesin vitro* and in fresh-cut lettuce. *Food Microbiology*, *24*, 759−766. http://dx.doi.org/10.1016/j.fm.2007.03.002.

Allende, A., Tomás-Barberán, F. A., & Gil, M. I. (2006). Minimal processing for healthy traditional foods. *Trends in Food Science & Technology*, *17*, 513−519. http://dx.doi.org/10.1016/j.tifs.2006.04.005.

Alvarez, M. V., Moreira, M. R., & Ponce, A. (2012). Antiquorum sensing and antimicrobial activity of natural agents with potential use in food. *Journal of Food Safety*, *32*, 379−387. http://dx.doi.org/10.1111/j.1745-4565.2012.00390.x.

Alvarez, M. V., Ponce, A. G., & Moreira, M. d. R. (2013). Antimicrobial efficiency of chitosan coating enriched with bioactive compounds to improve the safety of fresh cut broccoli. *LWT-Food Science and Technology*, *20*, 78−87. http://dx.doi.org/10.1016/j.lwt.2012.06.021.

Ayala-Zavala, J. F., Del Toro-Sánchez, L., Alvarez-Parrilla, E., & González-Aguilar, G. A. (2008). High relative humidity in-package of fresh-cut fruits and vegetables: advantage or disadvantage considering microbiological problems and antimicrobial delivering systems? *Journal of Food Science*, *73*, 41−47. http://dx.doi.org/10.1111/j.1750-3841.2008.00705.x.

Ayala-Zavala, J. F., & González-Aguilar, G. A. (2010). Optimizing the use of garlic oil as antimicrobial agent on fresh-cut tomato through a controlled release system. *Journal of Food Science*, *75*, 398−405. http://dx.doi.org/10.1111/j.1750-3841.2010.01723.x.

Ayala-Zavala, J. F., González-Aguilar, G. A., & Del Toro-Sánchez, L. (2009). Enhancing safety and aroma appealing of fresh-cut fruits and vegetables using the antimicrobial and aromatic

power of essential oils. *Journal of Food Science*, *74*, 84−91. http://dx.doi.org/10.1111/j.1750-3841.2009.01294.x.

Ayala-Zavala, J. F., Oms-Oliu, G., Odriozola-Serrano, I., González-Aguilar, G. A., Álvarez-Parrilla, E., & Martín-Belloso, O. (2008). Bio-preservation of fresh-cut tomatoes using natural antimicrobials. *European Food Research and Technology*, *226*, 1047−1055. http://dx.doi.org/10.1007/00217-007-0630-z.

Ayala-Zavala, J. F., Rosas-Domínguez, C., Vega-Vega, V., & González-Aguilar, G. A. (2010). Antioxidant enrichment and antimicrobial protection of fresh-cut fruits using their own byproducts: looking for integral exploitation. *Journal of Food Science*, *75*, 175−181. http://dx.doi.org/10.1111/j.1750-3841.2010.01792.x.

Ayala-Zavala, J. F., Silva-Espinoza, B. A., Cruz-Valenzuela, M. R., Leyva, J. M., Ortega-Ramírez, L. A., Carrazco-Lugo, D. K., et al. (2013). Pectin-cinnamon leaf oil coatings add antioxidant and antibacterial properties to fresh-cut peach. *Flavour and Fragrance Journal*, *28*, 39−45. http://dx.doi.org/10.1002/ffj.3125.

Ayala-Zavala, J. F., Wang, S. Y., Wang, C. Y., & González-Aguilar, G. A. (2005). Methyl jasmonate in conjunction with ethanol treatment increases antioxidant capacity, volatile compounds and postharvest life of strawberry fruit. *European Food Research and Technology*, *221*, 731−738. http://dx.doi.org/10.1007/s00217-005-0069-z.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils−a review. *Food and Chemical Toxicology*, *46*(2), 446−475. http://dx.doi.org/10.1016/j.fct.2007.09.106.

Bauer, K., Garbe, D., & Surburg, H. (2001). *Common fragrance and flavor materials* (4th ed.). Germany: Wiley-VCH Weinheim, 293.

Bayramoglu, B., Sahin, S., & Sumnu, G. (2008). Solvent-free microwave extraction of essential oil from oregano. *Journal of Food Engineering*, *88*(4), 535−540. http://dx.doi.org/10.1016/j.jfoodeng.2008.03.015.

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., et al. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology*, *12*(9), 2385−2397. http://dx.doi.org/10.1111/j.1462-2920.2010.02297.x.

Beuchat, L. R. (1996). *Listeria monocytogenes*: incidence on vegetables. *Food Control*, *7*(4), 223−228. http://dx.doi.org/10.1016/S0956-7135(96)00039-4.

Blasa, M., Gennari, L., Angelino, D., & Ninfali, P. (2010). Chapter 3-Fruit and vegetable antioxidants in health. In W. Ronald Ross, & R. P. Victor (Eds.), *Bioactive foods in promoting health* (pp. 37−58). San Diego: Academic Press.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*, 223−253. http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Chang, H.-T., Cheng, Y.-H., Wu, C.-L., Chang, S.-T., Chang, T.-T., & Su, Y.-C. (2008). Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* florin leaf against plant pathogenic fungi. *Bioresource Technology*, *99*, 6266−6270. http://dx.doi.org/10.1016/j.biortech.2007.12.005.

Chu, C.-L., Liu, W.-T., & Zhou, T. (2001). Fumigation of sweet cherries with thymol and acetic acid to reduce postharvest brown rot and blue mold rot. *Fruits*, *56*, 123−130. http://dx.doi.org/10.1051/fruits:2001113.

Chu, C.-L., Liu, W.-T., Zhou, T., & Tsao, R. (1999). Control of postharvest gray mold rot of modified atmosphere packaged sweet cherries by fumigation with thymol and acetic acid. *Canadian Journal of Plant Science*, *79*, 685−689. http://dx.doi.org/10.4141/P98-108.

Corbo, M. R., Lanciotti, R., Gardini, F., Sinigaglia, M., & Guerzoni, M. E. (2000). Effects of hexanal, trans-2-hexenal, and storage temperature on shelf life of fresh sliced apples.

Journal of Agricultural and Food Chemistry, *48*, 2401−2408. http://dx.doi.org/10.1021/jf991223f.

Corbo, M. R., Speranza, B., Campaniello, D., D'Amato, D., & Sinigaglia, M. (2010). Fresh-cut fruits preservation: current status and emerging technologies. In *Current research, technology and education topics in applied microbiology and microbial biotechnology* (Vol. 2). Badajoz: Formatex Research Center. pp. 1143−1154.

Cruz-Valenzuela, M. R., Carrazco-Lugo, D. K., Vega-Vega, V., Gonzalez-Aguilar, G. A., & Ayala-Zavala, J. F. (2013). Fresh−cut orange treated with its own seed by−products presented higher antioxidant capacity and lower microbial growth. *International Journal of Postharvest Technology and Innovation*, *3*(1), 13−27. http://dx.doi.org/10.1504/IJPTI.2013.051987.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*, *23*, 174−181. http://dx.doi.org/10.1016/j.copbio.2011.08.007.

Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annual Review of Food Science and Technology*, *4*, 163−190. http://dx.doi.org/10.1146/annurev-food-030212-182535.

Davidson, P. M., & Zivanovic, S. (2003). The use of natural antimicrobials. Ch. 2. In P. Zeuthen, & L. Bøgh-Sørensen (Eds.), *Food preservation techniques* (pp. 5−30). Boca Raton, FL: CRC Press.

De Azeredo, G. A., Stamford, T. L. M., Nunes, P. C., Gomes Neto, N. J., De Oliveira, M. E. G., & De Souza, E. L. (2011). Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. *Food Research International*, *44*, 1541−1548. http://dx.doi.org/10.1016/j.foodres.2011.04.012.

De Sousa, L. L., De Andrade, S. C. A., Athayde, A. J. A. A., De Oliveira, C. E. V., De Sales, C. V., Madruga, M. S., et al. (2013). Efficacy of *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils in combination to control postharvest pathogenic *Aspergilli* and autochthonous mycoflora in *Vitis labrusca* L. (table grapes). *International Journal of Food Microbiology*, *165*, 312−318. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.06.001.

De Sousa, J. P., De Azerêdo, G. A., De Araújo Torres, R., Da Silva Vasconcelos, M. A., Da Conceição, M. L., & De Souza, E. L. (2012). Synergies of carvacrol and 1,8-cineole to inhibit bacteria associated with minimally processed vegetables. *International Journal of Food Microbiology*, *154*, 145−151. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.026.

Dianez, F., Santos, M., Blanco, R., & Tello, J. C. (2002). Fungicide resistance in *Botrytis cinerea* isolates from strawberry crops in Huelva (southwestern Spain). *Phytoparasitica*, *30*, 529−534. http://dx.doi.org/10.1007/BF02979759.

Dimitrijević, S. I., Mihajlovski, K. R., Antonović, D. G., Milanović-Stevanović, M. R., & Mijin, D. Ž. (2007). A study of the synergistic antilisterial effects of a sub-lethal dose of lactic acid and essential oils from *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Origanum vulgare* L. *Food Chemistry*, *104*, 774−782. http://dx.doi.org/10.1016/j.foodchem.2006.12.028.

Eikani, M. H., Golmohammad, F., & Rowshanzamir, S. (2007). Subcritical water extraction of essential oils from coriander seeds (*Coriandrum sativum* L.). *Journal of Food Engineering*, *80*(2), 735−740. http://dx.doi.org/10.1016/j.jfoodeng.2006.05.015.

EPD. (2000). Strategic sewage disposal scheme − environmental impact assessment study. *Final Report. January 2000. Agreement CE 52/95. Submitted to Environmental Protection Department by Montgomery Watson and Binhai Wastewater Treatment & Disposal Consultants Ltd*. Hong Kong: Environmental Protection Department.

Feng, W., & Zheng, X. (2007). Essential oils to control *Alternaria alternata in vitro* and *in vivo*. *Food Control*, *18*, 1126−1130. http://dx.doi.org/10.1016/j.foodcont.2006.05.017.

Filip, V., Plockova, M., Šmidrkal, J., Špičková, Z., Melzoch, K., & Schmidt, Š. (2003). Resveratrol and its antioxidant and antimicrobial effectiveness. *Food Chemistry*, *83*(4), 585−593. http://dx.doi.org/10.1016/S0308-8146(03)00157-2.

Garrett, E. H., Gorny, J. R., Beuchat, L. R., Farber, J. N., Harris, L. J., Parish, M. E., et al. (2003). Microbiological safety of fresh and fresh-cut produce: description of the situation and economic impact. *Comprehensive Reviews in Food Science and Food Safety*, *2*, 13−37. http://dx.doi.org/10.1111/j.1541-4337.2003.tb00029.x.

Goñi, M. G., Moreira, M. R., Viacava, G. E., & Roura, S. I. (2013a). Optimization of chitosan treatments for managing microflora in lettuce seeds without affecting germination. *Carbohydrate Polymers*, *92*, 817−823. http://dx.doi.org/10.1016/j.carbpol.2012.09.094.

Goñi, M. G., Tomadoni, B., Moreira, M. R., & Roura, S. I. (2013). Application of tea tree and clove essential oil on late development stages of Butterhead lettuce: impact on microbiological quality. *LWT - Food Science and Technology*, *1*, 107−113. http://dx.doi.org/10.1016/j.lwt.2013.04.021.

González-Aguilar, G., Tiznado-Hernández, M., & Wang, C. Y. (2006). Physiological and biochemical responses of horticultural products to methyl jasmonate. *Stewart Postharvest Review*, *2*, 1−9. http://dx.doi.org/10.2212/spr.2006.1.1.

González-Aguilar, G. A., Valenzuela-Soto, E., Lizardi-Mendoza, J., Goycoolea, F., Martínez-Téllez, M. A., Villegas-Ochoa, M. A., et al. (2009). Effect of chitosan coating in preventing deterioration and preserving the quality of fresh-cut papaya 'Maradol'. *Journal of the Science of Food and Agriculture*, *89*, 15−23. http://dx.doi.org/10.1002/jsfa.3405.

Guan, W., Li, S., Yan, R., Tang, S., & Quan, C. (2007). Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chemistry*, *101*(4), 1558−1564. http://dx.doi.org/10.1016/j.foodchem.2006.04.009.

Gutierrez, J., Bourke, P., Lonchamp, J., & Barry-Ryan, C. (2009). Impact of plant essential oils on microbiological, organoleptic and quality markers of minimally processed vegetables. *Innovative Food Science & Emerging Technologies*, *10*(2), 195−202. http://dx.doi.org/10.1016/j.ifset.2008.10.005.

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., et al. (2003). Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprenhensive Reviews in Food Science and Food Safety*, *2*, 78−141. http://dx.doi.org/10.1111/j.1541-4337.2003.tb00031.x.

Jiménez, J. B., Orea, J. M., Montero, C., Ureña, A. G., Navas, E., Slowing, K., et al. (2005). Resveratrol treatment controls microbial flora, prolongs shelf life, and preserves nutritional quality of fruit. *Journal of Agricultural and Food Chemistry*, *53*, 1526−1530. http://dx.doi.org/10.1021/jf048426a.

Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunology and Cell Biology*, *78*(1), 80−88. http://dx.doi.org/10.1046/j.1440-1711.2000.00886.x.

Kim, S.-Y., Kang, D.-H., Kim, J.-K., Ha, Y.-G., Hwang, J. Y., Kim, T., et al. (2011). Antimicrobial activity of plant extracts against *Salmonella* Typhimurium, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on fresh lettuce. *Journal of Food Science*, *76*, 41−46. http://dx.doi.org/10.1111/j.1750-3841.2010.01926.x.

Krasaekoopt, W., Bhandari, B., Sinha, N., & Hui, Y. (2011). 'Fresh-cut vegetables', Ch. 10. In N. K. Sinha, Y. H. Hui, E.Ö. Evranuz, M. Siddiq, & J. Ahmed (Eds.), *Handbook of vegetables and vegetable processing* (pp. 221−242). Iowa, USA: Wiley-Blackwell.

Kumar, A., Shukla, R., Singh, P., Prasad, C. S., & Dubey, N. K. (2008). Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. *Innovative Food Science & Emerging Technologies*, *9*, 575–580. http://dx.doi.org/10.1016/j.ifset.2007.12.005.

Lanciotti, R., Belletti, N., Patrignani, F., Gianotti, A., Gardini, F., & Guerzoni, M. E. (2003). Application of hexanal,(E)-2-hexenal, and hexyl acetate to improve the safety of fresh-sliced apples. *Journal of Agricultural and Food Chemistry*, *51*, 2958–2963. http://dx.doi.org/10.1021/jf026143h.

Lanciotti, R., Corbo, M. R., Gardini, F., Sinigaglia, M., & Guerzoni, M. E. (1999). Effect of hexanal on the shelf life of fresh apple slices. *Journal of Agricultural and Food Chemistry*, *47*, 4769–4776. http://dx.doi.org/10.1021/jf990611e.

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M. E., & Gardini, F. (2004). Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends in Food Science & Technology*, *15*, 201–208. http://dx.doi.org/10.1016/j.tifs.2003.10.004.

Lee, S.-Y., Costello, M., & Kang, D.-H. (2004). Efficacy of chlorine dioxide gas as a sanitizer of lettuce leaves. *Journal of Food Protection*, *67*, 1371–1376.

Liu, N., Chen, X.-G., Park, H.-J., Liu, C.-G., Liu, C.-S., Meng, X.-H., et al. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymers*, *64*, 60–65. http://dx.doi.org/10.1016/j.carbpol.2005.10.028.

Lopez-Reyes, J. G., Spadaro, D., Gullino, M. L., & Garibaldi, A. (2010). Efficacy of plant essential oils on postharvest control of rot caused by fungi on four cultivars of apples *in vivo*. *Flavour and Fragrance Journal*, *25*, 171–177. http://dx.doi.org/10.1002/ffj.1989.

Luciano, F. B., & Holley, R. A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *International Journal of Food Microbiology*, *131*, 240–245. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.03.005.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*, 727–747.

Mari, M., Leoni, O., Bernardi, R., Neri, F., & Palmieri, S. (2008). Control of brown rot on stonefruit by synthetic and glucosinolate-derived isothiocyanates. *Postharvest Biology and Technology*, *47*, 61–67. http://dx.doi.org/10.1016/j.postharvbio.2007.06.003.

Mari, M., Leoni, O., Iori, R., & Cembali, T. (2002). Antifungal vapour-phase activity of allyl-isothiocyanate against *Penicillium expansum* on pears. *Plant Pathology*, *51*, 231–236. http://dx.doi.org/10.1046/j.1365-3059.2002.00667.x.

Martín-Diana, A. B., Rico, D., & Barry-Ryan, C. (2008). Green tea extract as a natural antioxidant to extend the shelf-life of fresh-cut lettuce. *Innovative Food Science & Emerging Technologies*, *9*, 593–603. http://dx.doi.org/10.1016/j.ifset.2008.04.001.

Martínez-Ferrer, M., & Harper, C. (2005). Reduction in microbial growth and improvement of storage quality in fresh-cut pineapple after methyl jasmonate treatment. *Journal of Food Quality*, *28*, 3–12. http://dx.doi.org/10.1111/j.1745-4557.2005.00007.x.

Moreira, M. R., Ponce, A. G., del Valle, C. E., & Roura, S. I. (2005). Inhibitory parameters of essential oils to reduce a foodborne pathogen. *LWT - Food Science and Technology*, *38*, 565–570. http://dx.doi.org/10.1016/j.lwt.2004.07.012.

Moreira, M. R., Ponce, A. G., Del Valle, C. E., & Roura, S. I. (2007). Effects of clove and tea tree oils on *Escherichia coli* O157:H7 in blanched spinach and minced cooked beef. *Journal of Food Processing and Preservation*, *31*, 379–391. http://dx.doi.org/10.1111/j.1745-4549.2007.00135.x.

Moss, M. O. (2008). Fungi, quality and safety issues in fresh fruits and vegetables. *Journal of Applied Microbiology*, *104*(5), 1239−1243. http://dx.doi.org/10.1111/j.1365-2672.2007.03705.x.

Mostowy, S., & Cossart, P. (2012). 'Virulence factors that modulate the cell biology of *Listeria* infection and the host response', Ch. 3. In E. T. Unanue, & J. A. Carrero (Eds.), *Advances in immunology: immunity to Listeria monocytogenes* (pp. 19−32). Elsevier Inc.

Ngarmsak, M., Delaquis, P., Toivonen, P., Ngarmsak, T., Ooraikul, B., & Mazza, G. (2006). Antimicrobial activity of vanillin against spoilage microorganisms in stored fresh-cut mangoes. *Journal of Food Protection*, *69*, 1724−1727.

Obrig, T. G. (2010). *Escherichia coli* Shiga toxin mechanisms of action in renal disease. *Toxins*, *2*(12), 2769−2794. http://dx.doi.org/10.3390/toxins2122769.

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., et al. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998−2008. *Emerging Infectious Diseases*, *19*(3), 407. http://dx.doi.org/10.3201/eid1903.111866.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—potential applications in food safety and quality. *Food Research International*, *44*, 827−839. http://dx.doi.org/10.1016/j.foodres.2011.01.022.

Pirola, L., & Fröjdö, S. (2008). Resveratrol: one molecule, many targets. *IUBMB Life*, *60*, 323−332. http://dx.doi.org/10.1002/iub.47.

Ponce, A. G., Del Valle, C., & Roura, S. I. (2004a). Shelf life of leafy vegetables treated with natural essential oils. *Journal of Food Science*, *69*, 50−56. http://dx.doi.org/10.1111/j.1365-2621.2004.tb15515.x.

Ponce, A. G., Del Valle, C. E., & Roura, S. I. (2004b). Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. *LWT - Food Science and Technology*, *37*, 199−204. http://dx.doi.org/10.1016/j.lwt.2003.07.005.

Ponce, A., Roura, S. I., & Moreira, M. d. R. (2011). Essential oils as biopreservatives: different methods for the technological application in lettuce leaves. *Journal of Food Science*, *76*, 34−40. http://dx.doi.org/10.1111/j.1750-3841.2010.01880.x.

Rabanal, R. M., Arias, A., Prado, B., Hernández-Pérez, M., & Sánchez-Mateo, C. C. (2002). Antimicrobial studies on three species of *Hypericum* from the Canary Islands. *Journal of Ethnopharmacology*, *81*, 287−292. http://dx.doi.org/10.1016/S0378-8741(02)00083-1.

Ragaert, P., Devlieghere, F., & Debevere, J. (2007). Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. *Postharvest Biology and Technology*, *44*(3), 185−194. http://dx.doi.org/10.1016/j.postharvbio.2007.01.001.

Ragaert, P., Jacxsens, J., Vandekinderen, I., Baert, L., & Devlieghere, F. (2010). Microbiological and safety aspects of fresh-cut fruits and vegetables. Ch. 3. In O. M. Belloso, & R. S. Fortuny (Eds.), *Advances in fresh-cut fruits and vegetables processing* (pp. 53−86). Boca Ratón, FL: CRC Press, Taylor & Francis Inc.

Ragaert, P., Verbeke, W., Devlieghere, F., & Debevere, J. (2004). Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Quality and Preference*, *15*, 259−270. http://dx.doi.org/10.1016/S0950-3293(03)00066-1.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., et al. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology*, *56*, 3−12. http://dx.doi.org/10.1016/S0168-1605(00)00218-X.

Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., Soliva-Fortuny, R., & Martín-Belloso, O. (2009). Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit

juices by traditional and alternative natural antimicrobials. *Comprehensive Reviews in Food Science and Food Safety*, *8*, 157−180. http://dx.doi.org/10.1111/j.1541-4337.2009.00076.x.

Rico, D., Martín-Diana, A. B., Barat, J. M., & Barry-Ryan, C. (2007). Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science & Technology*, *18*, 373−386. http://dx.doi.org/10.1016/j.tifs.2007.03.011.

Rojas-Graü, M. A., Raybaudi-Massilia, R. M., Soliva-Fortuny, R. C., Avena-Bustillos, R. J., McHugh, T. H., & Martín-Belloso, O. (2007). Apple puree-alginate edible coating as carrier of antimicrobial agents to prolong shelf-life of fresh-cut apples. *Postharvest Biology and Technology*, *45*, 254−264. http://dx.doi.org/10.1016/j.postharvbio.2007.01.017.

Roller, S., & Seedhar, P. (2002). Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4° and 8°C. *Letters in Applied Microbiology*, *35*, 309−394. http://dx.doi.org/10.1046/j.1472-765X.2002.01209.x.

Rupasinghe, H. P., Boulter-Bitzer, J., Ahn, T., & Odumeru, J. A. (2006). Vanillin inhibits pathogenic and spoilage microorganisms *in vitro* and aerobic microbial growth in fresh-cut apples. *Food Research International*, *39*, 575−580. http://dx.doi.org/10.1016/j.foodres.2005.11.005.

Rydlo, T., Miltz, J., & Mor, A. (2006). Eukaryotic antimicrobial peptides: promises and premises in food safety. *Journal of Food Science*, *71*, 125−135. http://dx.doi.org/10.1111/j.1750-3841.2006.00175.x.

Sebti, I., Martial-Gros, A., Carnet-Pantiez, A., Grelier, S., & Coma, V. (2005). Chitosan polymer as bioactive coating and film against *Aspergillus niger* contamination. *Journal of Food Science*, *70*, 100−104. http://dx.doi.org/10.1111/j.1365-2621.2005.tb07098.x.

Siddiqui, MdW., Chakraborty, I., Ayala-Zavala, J. F., & Dhua, R. S. (2011). Advances in minimal processing of fruits and vegetables: a review. *Journal of Scientific & Industrial Research*, *70*, 823−834.

Singh, N., Singh, R. K., Bhunia, A. K., & Stroshine, R. L. (2002). Efficacy of chlorine dioxide, ozone, and thyme essential oil or a sequential washing in killing *Escherichia coli* O157: H7 on lettuce and baby carrots. *LWT-Food Science and Technology*, *35*, 720−729. http://dx.doi.org/10.1006/fstl.2002.0933.

Skandamis, P. N., & Nychas, G.-J. E. (2000). Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Applied and Environmental Microbiology*, *66*, 1646−1653. http://dx.doi.org/10.1128/AEM.66.4.1646-1653.2000.

Sterzenbach, T., Crawford, R. W., Winter, S. E., & Bäumler, A. J. (2013). Salmonella virulence mechanisms and their genetic basis. Ch. 5. In P. A. Barrow, & U. Methner (Eds.), *Salmonella in Domestic animals* (pp. 80−103). Wallingford, UK: CABI Publishing.

Trias, R., Bañeras, L., Badosa, E., & Montesinos, E. (2008). Bioprotection of golden delicious apples and iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International Journal of Food Microbiology*, *123*, 50−60. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.11.065.

Tripathi, P., & Dubey, N. K. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, *32*, 235−245. http://dx.doi.org/10.1016/j.postharvbio.2003.11.005.

Tripathi, P., Dubey, N. K., & Shukla, A. K. (2008). Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. *World Journal of Microbiology and Biotechnology*, *24*, 39−46. http://dx.doi.org/10.1007/s11274-007-9435-2.

Tzortzakis, N. G. (2009). Impact of cinnamon oil-enrichment on microbial spoilage of fresh produce. *Innovative Food Science & Emerging Technologies*, *10*, 97−102. http://dx.doi.org/10.1016/j.ifset.2008.09.002.

Tzortzakis, N. G., & Economakis, C. D. (2007). Antifungal activity of lemongrass (*Cympopogon citratus* L.) essential oil against key postharvest pathogens. *Innovative Food Science & Emerging Technologies*, *8*, 253−258. http://dx.doi.org/10.1016/j.ifset.2007.01.002.

Ukuku, D. O., & Fett, W. F. (2002). Effectiveness of chlorine and nisin-EDTA treatments of whole melons and fresh-cut pieces for reducing native microflora and extending shelf-life. *Journal of Food Safety*, *22*, 231−253. http://dx.doi.org/10.1111/j.1745-4565.2002.tb00344.x.

Ukuku, D. O., & Fett, W. F. (2004). Effect of nisin in combination with EDTA, sodium lactate, and potassium sorbate for reducing *Salmonella* on whole and fresh-cut cantaloupet. *Journal of Food Protection*, *67*, 2143−2150.

Utama, I. M. S., Wills, R. B., Ben-yehoshua, S., & Kuek, C. (2002). *In vitro* efficacy of plant volatiles for inhibiting the growth of fruit and vegetable decay microorganisms. *Journal of Agricultural and Food Chemistry*, *50*, 6371−6377. http://dx.doi.org/10.1021/jf020484d.

Vega-Vega, V., Silva-Espinoza, B. A., Cruz-Valenzuela, M. R., Bernal-Mercado, A. T., González-Aguilar, G. A., Vargas-Arispuro, I., et al. (2013). Antioxidant enrichment and antimicrobial protection of fresh-cut mango applying bioactive extracts from their seeds by-products. *Food and Nutrition Sciences*, *4*, 197−203. http://dx.doi.org/10.4236/fns.2013.48A024.

Wang, C. Y., & Buta, J. G. (2003). Maintaining quality of fresh-cut kiwifruit with volatile compounds. *Postharvest Biology and Technology*, *28*, 181−186. http://dx.doi.org/10.1016/S0925-5214(02)00125-4.

Wan, J., Wilcock, A., & Coventry, M. J. (1998). The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *Journal of Applied Microbiology*, *84*, 152−158.

Weissinger, W. R., McWatters, K. H., & Beuchat, L. R. (2001). Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. *Journal of Food Protection*, *64*, 442−450.

Wilson, A. E., Bergaentzlé, M., Bindler, F., Marchioni, E., Lintz, A., & Ennahar, S. (2013). *In vitro* efficacies of various isothiocyanates from cruciferous vegetables as antimicrobial agents against foodborne pathogens and spoilage bacteria. *Food Control*, *30*, 318−324. http://dx.doi.org/10.1016/j.foodcont.2012.07.031.

Wu, H., Zhang, X., Zhang, G.-A., Zeng, S.-Y., & Lin, K.-C. (2011). Antifungal vapour-phase activity of a combination of allyl isothiocyanate and ethyl isothiocyanate against *Botrytis cinerea* and *Penicillium expansum* infection on apples. *Journal of Phytopathology*, *159*, 450−455. http://dx.doi.org/10.1111/j.1439-0434.2011.01792.x.

Xu, W., Qu, W., Huang, K., Guo, F., Yang, J., Zhao, H., et al. (2007). Antibacterial effect of grapefruit seed extract on food-borne pathogens and its application in the preservation of minimally processed vegetables. *Postharvest Biology and Technology*, *45*, 126−133. http://dx.doi.org/10.1016/j.postharvbio.2006.11.019.

14 Using natural antimicrobials to enhance the safety and quality of fresh and processed fruits and vegetables: application techniques and quality issues

M.V. Alvarez[1], *M. del R. Moreira*[1], *S.I. Roura*[1], *J.F. Ayala-Zavala*[2], *G.A. González-Aguilar*[2]
[1]Universidad Nacional de Mar del Plata, Buenos Aires, Argentina; [2]Centro de Investigación en Alimentación y Desarrollo, Hermosillo, Mexico

14.1 Introduction

Building on the previous chapter, which looked at the types of natural antimicrobial that could be used to preserve fresh and processed fruits and vegetables, this chapter reviews factors affecting the effectiveness of natural antimicrobials and techniques for their application to fruits and vegetables. It also discusses the antioxidant and flavor-enhancement properties of natural antimicrobials, improving product quality as well as safety.

14.2 Techniques for applying natural antimicrobials to fruits and vegetables: key issues

When designing the application of an antimicrobial to a food, some critical aspects that may affect the effectiveness of the additive as a preservative should be taken into account. These factors relate to the type of antimicrobial (physicochemical properties), food type (composition), processing operations to which it was subjected, conditions in which it will be stored, and target microorganism or microbial population. For the application of an additive to be viable, it is very important to evaluate the impact of its application on the sensory characteristics of the food. As was mentioned earlier, many natural antimicrobials, extracts, or natural compounds can transfer odors and flavors to the food. In some cases, these odors and flavors can be pleasant, but in others extremely unpleasant, to the consumer. Therefore, it is necessary that the potential additive is compatible with the food from the sensory point of view (Ayala-Zavala, González-Aguilar, & Del Toro-Sánchez, 2009).

Also, it has to be taken into account that the composition of the food to be treated influences the activity of the antimicrobial additive. It is common for antimicrobials

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that prove very effective by *in vitro* assays lose effectiveness when applied to a food matrix. Thus, higher concentrations have to be used in order to obtain a preservative effect. This decrease of the antimicrobial activity can be attributed to interactions between the compounds present in the additive and the food components. It has been shown that proteins, lipids, complex carbohydrates, and sugars reduce antimicrobial activity (Devlieghere, Vermeulen, & Debevere, 2004; Gutierrez, Barry-Ryan, & Bourke, 2008). The effective application of natural antimicrobials to fruits is not severely affected by interactions due to its low protein and lipids content, although the possible effects of interactions with carbohydrates should be taken into account. Also, in many fruits, lowered pH may act together with the antimicrobial in inhibiting the growth of microorganisms (Davidson, Critzer, & Taylor, 2013). Moreover, vegetables also have a low content of proteins and lipids, but they have higher pH and water activity than fruits, and so higher concentrations of antimicrobials may be required for their preservation (Davidson et al., 2013). It is also important to note that the effectiveness of the antimicrobials, specifically flavor compounds and essential oils, may be reduced not only by interactions with the food matrix but also because of their high volatility (Ayala-Zavala, Del Toro-Sánchez, Alvarez-Parrilla, & González-Aguilar, 2008a).

The forms of application of antimicrobials traditionally used by the fruit and vegetable industry include solutions or emulsions, applied by dipping or spraying the product. Dusting is also used when it comes to powders. New technologies have been developed and continue to be under study with the aim of solving the problems associated with the effective implementation of natural antimicrobials, as described above. In the face of the need to use concentrations that are sufficient to inhibit microbial growth but that at the same time allow the preservation of the sensory appeal of the products, technological developments focus on the incorporation of natural antimicrobials into edible films and coatings and also encapsulation of these agents in a food-grade material. Figure 14.1 summarizes the systems used to incorporate natural antimicrobials on fresh and processed produce.

14.3 Encapsulation of natural antimicrobials

Depending on the time and temperature of storage, volatile compounds present in essential oils and applied in foods can volatilize or become inactive (Ayala-Zavala et al., 2008a). Micro- and nanoencapsulation is defined as a technology for packaging solids, liquids, or gaseous substances in small particles (at a micro- and nanoscale) in capsules. The encapsulation of bioactive agents allows the stabilization of the compounds and reduces their degradation due to interactions with the components of the food. It is also used to protect compounds from environmental factors such as light and oxygen that may cause their degradation, and also it stabilizes volatile antimicrobials (Donsì, Annunziata, Sessa, & Ferrari, 2011). And the most important advantage of encapsulating compounds is that it is possible to regulate their release, resulting in a longer exposure of the microorganisms to the antimicrobial as well as a lower impact

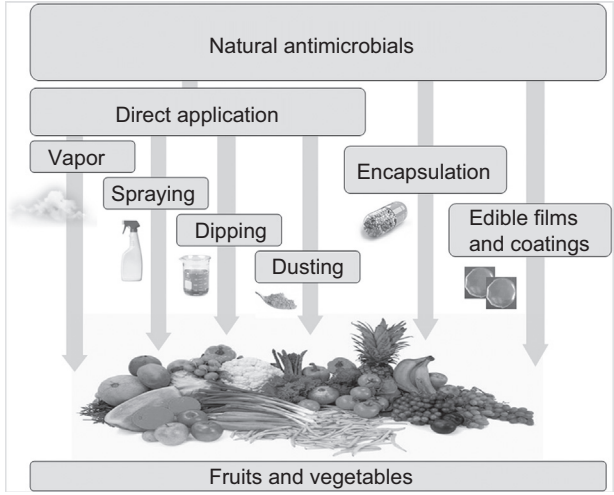

Figure 14.1 Systems to incorporate natural antimicrobials on fresh and processed produce.

in the sensory quality of the product. Encapsulation of essential oils in β-cyclodextrin is a method to control the reactivity and the odor caused by volatiles, allowing the controlled release of compounds (Ayala-Zavala et al., 2008b).

Some studies have demonstrated *in vitro* antifungal activity of thyme, cinnamon leaf, and garlic essential oils microencapsulated in β-cyclodextrin against *Alternaria alternata* to control decay of stored fruits and vegetables (Ayala-Zavala et al., 2008b; Del Toro-Sánchez et al., 2010). Garlic oil capsules (in β-cyclodextrin) and free garlic oil were tested on fresh-cut tomatoes to control microbial growth. Free oil (100 and 200 μg/100 g) applied to tomatoes inhibited microbial growth but did not present acceptable sensory qualities for panelists. However, the highest concentration of garlic oil capsules applied (1%) showed the lowest microbial growth and the highest sensory quality (Ayala-Zavala & González-Aguilar, 2010).

The encapsulation of essential oils and other agents into nanometric delivery systems is also a promising technology. Donsì et al. (2011) prepared nanoemulsions based on food-grade ingredients from a terpenes mixture (obtained from *Melaleuca alternifolia*) by high-pressure homogenization. The *in vitro* antimicrobial activity of the terpenes was enhanced by the nanoencapsulation process, compared with the unencapsulated mixture, against *Lactobacillus delbrueckii*, *Saccharomyces cerevisiae*, *and Escherichia coli*. Furthermore, the terpenes nanocapsules were tested in orange and pear juices inoculated with *Lactobacillus delbreuckii* and other spoilage bacteria. Lower terpene concentrations were required for a bactericidal action when they were delivered as nanocapsules. Complete inactivation of the microorganisms was achieved by applying 5.0 g/l of encapsulated terpenes, and the sensory quality of the fruit juices was minimally altered. In these terms, the encapsulation techniques could be used to facilitate the use of natural compounds as food antimicrobial agents.

14.4 Edible films and coatings enriched with natural antimicrobials

Edible films and coatings are applied on many fruits and vegetables to control moisture transfer, gas exchange, or to prevent oxidation processes. However, the new generation of edible films and coatings is being especially designed as a carrier of active ingredients as antimicrobials, antioxidants, vitamins, and nutraceuticals. These agents are incorporated with the aim of assuring safety, preventing microbiological and sensory spoilage, or enhancing nutritional attributes of the product (Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2009). The application of antimicrobial coatings presents several advantages over the direct application of antimicrobials on food. These coatings can be developed to reduce the diffusion rate from the coated surface inwards. In this way, the activity of the antimicrobial agent is maintained in the surface of the food. Thus, smaller amounts of the antimicrobial come into contact with the food compared to other application methods such as immersion or spray (Min & Krochta, 2005). This results in a smaller impact in the sensory attributes of fruits and vegetables when they are treated with antimicrobial coatings.

Edible films and coatings for fresh and processed fruits and vegetables can be formulated from a wide variety of compounds, depending on the function to be fulfilled. The main components are polysaccharides (starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectin, and gums), proteins (casein and whey protein, wheat gluten, soy protein, and others), and lipids (e.g., fatty acids) (Vargas, Pastor, Chiralt, McClements, & González-Martínez, 2008). While there are several investigations that have studied the incorporation of antimicrobial agents into protein or polysaccharide-based coatings through *in vitro* assays, only a few formulations have been tested on fruits and vegetables for preservation. Thus, Ayala-Zavala et al. (2013) developed pectin films enriched with cinnamon leaf essential oil and applied on fresh-cut peaches to preserve their microbiological quality along with refrigerated shelf life. Enriched pectin film showed the highest antimicrobial activity at a cinnamon leaf oil concentration of 36.1 g/l. It was able to reduce significantly mesophilic bacteria growth, maintain overall quality and, increase shelf life of fresh-cut peaches.

The development of coatings from polymers that exhibit antimicrobial activity is very promising: such is the case of chitosan. Chitosan films can be used as a means to incorporate natural antimicrobials such as essential oils, plant extracts, nisin, and lysozyme (Alvarez, Ponce, & Moreira, 2013; Pranoto, Rakshit, & Salokhe, 2005; Zivanovic, Chi, & Draughon, 2005). With the aim to assure safety and extend shelf life of minimally processed broccoli, Alvarez et al. (2013) studied the antimicrobial properties of chitosan coatings combined with tea tree essential oil and with extracts rich in phenolic compounds like pomegranate peel extract, propolis extract, and resveratrol. They found that the enrichment of chitosan coatings with polyphenols enhanced antimicrobial action when compared with pure chitosan treatment. These treatments significantly reduced the growth of natural flora and pathogens (*E. coli* and *Listeria monocytogenes*) artificially inoculated on broccoli during refrigerated storage.

14.5 Antioxidant properties of natural antimicrobials

Many natural antimicrobials, such as essential oils, other volatile compounds, and also phenolic compounds, can act also as antioxidant agents at the same time that they are protecting the product against pathogens and spoilage microorganism's proliferation. Thus, the application of these agents to fresh and processed produce improves antioxidant content of the product, can avoid oxidative processes catalyzed by enzymes, and can also prevent losses of natural antioxidants such as ascorbic acid and carotenoids. Table 14.1 shows responses as antioxidant agents of several natural antimicrobials applied on fruits and vegetables.

Several studies have been developed applying natural compounds as antimicrobials and antioxidant additives on fresh and processed fruits and vegetables. Ayala-Zavala et al. (2013) reported that the treatment of fresh-cut peaches with an edible pectin film enriched with cinnamon leaf essential oil was effective to reduce bacterial growth and increase total phenolic content and antioxidant capacity of the product. This capacity was expressed as DPPH radical scavenging activity and trolox equivalent antioxidant capacity (TEAC) values. Furthermore, Wang, Wang, and Chen (2008) evaluated the effectiveness of several naturally occurring essential oils in reducing decay and increasing antioxidant levels and activities in "Duke" blueberries (*Vaccinium corymbosum*). Carvacrol, anethole, and perillaldehyde were able to promote total anthocyanins and total phenolics and also enhanced antioxidant activity in fruit tissues, expressed as oxygen radical absorbance capacity (ORAC) and hydroxyl radical (•OH) scavenging capacity. Also, these treatments showed the capability to inhibit fruit decay development compared to controls.

Green tea extract, mainly composed of polyphenols (cathechins and other flavonoids), was very well studied as an antimicrobial and antioxidant agent (Perumalla & Hettiarachchy, 2011). Martin-Diana, Rico, and Barry-Ryan (2008) applied this extract as a preservative treatment for fresh-cut lettuce. The use of green tea at the lowest concentration tested (0.25 g/100 ml) was effective to preserve the ascorbic acid and carotenoid content of the samples. However, the use of higher concentrations (0.5 or 1.0 g/100 ml) resulted in unacceptable products from a sensory point of view, due to the appearance of browning on lettuce leaves. Enzymatic browning caused by polyphenol oxidase (PPO) is a major detrimental factor of the quality of fresh-cut fruits and vegetables (Ayala-Zavala et al., 2011). Therefore, peroxidase (POD) is a plant enzyme associated with off-flavors and browning in fruits and vegetables (Ponce, Del Valle, & Roura, 2004b). Inactivation of these enzymes is considered necessary to minimize the possibility of deterioration. To avoid this problem, several additives have been applied mainly by dipping, spraying, or vacuum impregnation.

Chanjirakul, Wang, Wang, and Siriphanich (2006) studied the changes in antioxidant capacity and antioxidant enzyme activities in raspberries (*Rubus idaeus* L.) treated with several natural volatile compounds: methyl jasmonate, allyl isothiocyanate, tea tree essential oil, and ethanol. All of the natural compounds tested reduced

Table 14.1 Secondary responses of the treatment of fresh and processed produce treated with natural agents

Antimicrobial agent	Treated produce	Secondary responses	References
Cinnamon leaf essential oil in pectin film	Fresh-cut peaches	Increased total phenolics and antioxidant capacity; improved odor acceptability	Ayala-Zavala et al. (2013)
Carvacrol, anethole, and perillaldehyde	"Duke" blueberries	Increased total anthocyanins and total phenolics; enhanced antioxidant activity	Wang et al. (2008)
Green tea extract	Fresh-cut lettuce	Preserved ascorbic acid and carotenoid content; at high concentrations, affected sensorial acceptability due browning appearing	Martin-Diana et al. (2008)
Methyl jasmonate	Raspberries	Increased antioxidant capacity and increased activity in all antioxidant enzymes	Chanjirakul et al. (2006)
Clove, rosemary, lemon, melisa, and tea tree essential oils	Swiss chard, spinach, butter lettuce and cabbage	Reduced peroxidase activity	Ponce et al. (2004b)
Chitosan coatings enriched with rosemary and olive oleoresins	Fresh-cut butternut squash	Inhibited polyphenol oxidase and peroxidase activity	Ponce et al. (2008)
Lemongrass, oregano essential oil, and vanillin in edible coatings	Fresh-cut apple	Vanillin increased flavor quality, oregano affected it negatively	Rojas-Graü et al. (2007)

Table 14.1 Continued

Antimicrobial agent	Treated produce	Secondary responses	References
Tea tree, lemon, rosemary, and clove essential oils	Swiss chard	Affected odor immediately after application	Ponce et al. (2004a)
Hexanal, 2-(E)-hexenal and hexyl acetate	Fresh-cut apple	Enhanced sensorial properties and retained color	Lanciotti et al. (2004)

the severity of decay during storage at 10 °C when compared to the control. The methyl jasmonate treatment had the highest antioxidant capacity (expressed as ORAC) and also showed the highest activity in all antioxidant enzymes, including superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase. These results indicate that methyl jasmonate may increase the resistance of tissues to decay through enhancing their antioxidant system and their free-radical scavenging capability.

Antioxidant properties of several plant extracts were studied on vegetable products. Ponce et al. (2004b) demonstrated the efficacy of clove, rosemary, lemon, Melissa, and tea tree essential oils as reducing agents of peroxidase activity in leafy vegetables (swiss chard, spinach, lettuce, butter lettuce, and cabbage). Essential oils were used at the minimum inhibitory concentration determined in previous *in vitro* antimicrobial assays. Clove essential oil showed better results than the others. Impact of treatments on the sensory quality of vegetables was not reported in this study. Also, Ponce, Roura, Del Valle, and Moreira (2008) evaluated antimicrobial and antioxidant properties of several film-forming solutions, alone and enriched with oleoresins, applied by immersion, on butternut squash slices. The antioxidant properties of the film-forming solutions and the oleoresins were determined analyzing POD and PPO activities in extracts prepared from the treated butternut squash. The use of chitosan coatings enriched with rosemary and olive oleoresins did not produce a significant antimicrobial effect; however, antioxidant activity was observed the first day, exerting POD inhibition for up to 5 days of storage. Oleoresins alone and combined with chitosan exerted significant antioxidant activities over PPO through 5 days of storage. Also, it was demonstrated that treatments did not affect the sensorial acceptability of the product. Finally, these authors affirmed that these treatments were effective for the prevention of browning reactions, which typically result in quality loss in fruits and vegetables.

14.6 Plant antimicrobials as flavoring compounds

As described earlier, essential oils and volatile compounds can be used to assure safety and prevent decay in fresh and processed fruit and vegetables. However, it is necessary to consider that their volatile constituents could be absorbed by the produce, and according to the aroma notes of the antimicrobial oil, the sensory appeal of odor and flavor of the treated produce might be affected positively or negatively (Ayala-Zavala et al., 2009). Several studies evaluated the impact of a variety of natural antimicrobials on sensory quality of treated fruits and vegetables. Some of these results are shown in Table 14.1.

Recently, Ayala-Zavala et al. (2013) demonstrated that the treatment of fresh-cut peaches with pectin films enriched with cinnamon leaf essential oil was effective to control bacterial growth and also to improve odor acceptability of the product compared to untreated controls at day 10 of storage at 5 °C. It can be concluded that this treatment exhibited compatibility with the food from a sensory point of view. In addition, alginate and gellan edible coatings have been tested in combination with antimicrobial essential oils (lemongrass, oregano oil, and vanillin) to extend shelf life of fresh-cut apples. Treatments with apple puree—alginate enriched with lemongrass and oregano showed bactericidal effect against *Listeria innocua* inoculated in apples (Rojas-Graü et al., 2007). A sensorial study to evaluate the effect of the treatments on the flavor of fresh-cut apples revealed that coated apples containing vanillin (0.3% w/w) were the most acceptable in terms of flavor quality after 2 weeks of storage. On the other hand, apples treated with oregano, even at low concentration (0.1% w/w), had the lowest acceptability due to residual herbal taste detected by consumers in the product.

Many studies demonstrated that essential oils, applied in high concentrations, can negatively affect consumer acceptability. In such cases other strategies can be considered, such as the search for synergistic combinations between essential oils or combined application of antimicrobials with other factors in hurdle technologies. These strategies would help to achieve a well-balanced product, satisfying the antimicrobial and acceptability aspects of consumers' requirements (Ponce, Roura, & Moreira, 2011). In another study, Ponce, Del Valle, and Roura (2004a) studied antimicrobial and sensorial impact of eucalyptus, tea tree, lemon, rosemary, and clove essential oils applied as natural sanitizing agents on Swiss chard leaves. Although some of the essential oils appeared to reduce microbial counts, they were not effective in extending the shelf life of the Swiss chard leaves from a sensory point of view. Off odors were detected by the panelists in oil-treated samples right after spraying. After 1 day and up to day 14 of storage, however, the panelists did not find differences in odor among samples. If an antimicrobial agent possesses high effectiveness inhibiting microbial growth but negatively affects flavor and odor acceptability of produce, it would be unacceptable and definitely not viable to be applied. However, it should be considered that it is possible to improve safety and sensory appeal of a product at the same time by the application of essential oils. The key is selecting flavor compounds that are compatible with the fruit or vegetable to be treated. In this regard, Ayala-Zavala et al. (2009) described the aroma notes (sweet, herb, spice, and others)

of several fresh-cut fruits and vegetables and listed the possible combinations with essential oils that produced compatible results.

14.7 Conclusion and future trends

The incorporation of natural antimicrobials with fruits and vegetables without negatively affecting the sensory properties is still a challenge for researchers, since required concentrations to inhibit proliferation or eliminate pathogenic bacteria are several times higher than those accepted by consumers from a sensory point of view. Hence, combining these treatments with physical barriers, such as ultrasound and high pressures, constitutes a viable strategy to ensure the safety and nutritional and sensory quality of fresh produce. Furthermore, the research about potential synergistic combinations of natural agents to reduce microbial growth in fruits and vegetables is still scarce; this approach allows the use of lower concentrations, minimizing the effect on organoleptic characteristics of the treated food. Besides, multilayered edible coatings, replacing single-layer coatings, and also micro and nanoencapsulation, have proven to be promising technologies in the yield of fruits and vegetables, as they can be specially designed to incorporate and allow the controlled release of natural antimicrobials as well as antioxidants and other functional ingredients.

References

Alvarez, M. V., Ponce, A. G., & Moreira, M. d. R. (2013). Antimicrobial efficiency of chitosan coating enriched with bioactive compounds to improve the safety of fresh cut broccoli. *LWT—Food Science and Technology*, *20*, 78—87. http://dx.doi.org/10.1016/j.lwt.2012.06.021.

Ayala-Zavala, J. F., Del Toro-Sánchez, L., Alvarez-Parrilla, E., & González-Aguilar, G. A. (2008a). High relative humidity in-package of fresh-cut fruits and vegetables: advantage or disadvantage considering microbiological problems and antimicrobial delivering systems? *Journal of Food Science*, *73*, 41—47. http://dx.doi.org/10.1111/j.1750-3841.2008.00705.x.

Ayala-Zavala, J. F., & González-Aguilar, G. A. (2010). Optimizing the use of garlic oil as antimicrobial agent on fresh-cut tomato through a controlled release system. *Journal of Food Science*, *75*, 398—405. http://dx.doi.org/10.1111/j.1750-3841.2010.01723.x.

Ayala-Zavala, J. F., González-Aguilar, G. A., & Del Toro-Sánchez, L. (2009). Enhancing safety and aroma appealing of fresh-cut fruits and vegetables using the antimicrobial and aromatic power of essential oils. *Journal of Food Science*, *74*, 84—91. http://dx.doi.org/10.1111/j.1750-3841.2009.01294.x.

Ayala-Zavala, J. F., Silva-Espinoza, B. A., Cruz-Valenzuela, M. R., Leyva, J. M., Ortega-Ramírez, L. A., Carrazco-Lugo, D. K., et al. (2013). Pectin-cinnamon leaf oil coatings add antioxidant and antibacterial properties to fresh-cut peach. *Flavour and Fragrance Journal*, *28*, 39—45. http://dx.doi.org/10.1002/ffj.3125.

Ayala-Zavala, J. F., Soto-Valdez, H., González-León, A., Álvarez-Parrilla, E., Martín-Belloso, O., & González-Aguilar, G. A. (2008b). Microencapsulation of cinnamon leaf (*Cinnamomum zeylanicum*) and garlic (*Allium sativum*) oils in β-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *60*, 359—368. http://dx.doi.org/10.1007/s10847-007-9385-1.

Ayala-Zavala, J. F., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J. A., Siddiqui, Md W., et al. (2011). Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Research International*, *44*, 1866−1874. http://dx.doi.org/10.1016/j.foodres.2011.02.021.

Chanjirakul, K., Wang, S. Y., Wang, C. Y., & Siriphanich, J. (2006). Effect of natural volatile compounds on antioxidant capacity and antioxidant enzymes in raspberries. *Postharvest Biology and Technology*, *40*, 106−115. http://dx.doi.org/10.1016/j.postharvbio.2006.01.004.

Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annual Review of Food Science and Technology*, *4*, 163−190. http://dx.doi.org/10.1146/annurev-food-030212-182535.

Del Toro-Sánchez, C. L., Ayala-Zavala, J. F., Machi, L., Santacruz, H., Villegas-Ochoa, M. A., Álvarez-Parrilla, E., et al. (2010). Controlled release of antifungal volatiles of thyme essential oil from β-cyclodextrin capsules. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *67*, 431−441. http://dx.doi.org/10.1007/s10847-009-9726-3.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703−714. http://dx.doi.org/10.1016/j.fm.2004.02.008.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT − Food Science and Technology*, *44*, 1908−1914. http://dx.doi.org/10.1016/j.lwt.2011.03.003.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, *124*, 91−97. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.02.028.

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M. E., & Gardini, F. (2004). Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends in Food Science & Technology*, *15*, 201−208. http://dx.doi.org/10.1016/j.tifs.2003.10.004.

Martín-Diana, A. B., Rico, D., & Barry-Ryan, C. (2008). Green tea extract as a natural antioxidant to extend the shelf-life of fresh-cut lettuce. *Innovative Food Science & Emerging Technologies*, *9*, 593−603. http://dx.doi.org/10.1016/j.ifset.2008.04.001.

Min, S., & Krochta, J. M. (2005). Inhibition of *Penicillium commune* by edible whey protein films incorporating lactoferrin, lacto-ferrin hydrolysate, and lactoperoxidase systems. *Journal of Food Science*, *70*, 87−94. http://dx.doi.org/10.1111/j.1365-2621.2005.tb07108.x.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—potential applications in food safety and quality. *Food Research International*, *44*, 827−839. http://dx.doi.org/10.1016/j.foodres.2011.01.022.

Ponce, A. G., Del Valle, C., & Roura, S. I. (2004a). Shelf life of leafy vegetables treated with natural essential oils. *Journal of Food Science*, *69*, 50−56. http://dx.doi.org/10.1111/j.1365-2621.2004.tb15515.x.

Ponce, A. G., Del Valle, C. E., & Roura, S. I. (2004b). Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. *LWT − Food Science and Technology*, *37*, 199−204. http://dx.doi.org/10.1016/j.lwt.2003.07.005.

Ponce, A. G., Roura, S. I., Del Valle, C. E., & Moreira, M. R. (2008). Antimicrobial and antioxidant activities of edible coatings enriched with natural plant extracts: *in vitro* and *in vivo* studies. *Postharvest Biology and Technology*, *49*, 294−300. http://dx.doi.org/10.1016/j.postharvbio.2008.02.013.

Ponce, A., Roura, S. I., & Moreira, M. d. R. (2011). Essential oils as biopreservatives: different methods for the technological application in lettuce leaves. *Journal of Food Science*, *76*, 34−40. http://dx.doi.org/10.1111/j.1750-3841.2010.01880.x.

Pranoto, Y., Rakshit, S. K., & Salokhe, V. M. (2005). Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT − Food Science and Technology*, *38*, 859−865. http://dx.doi.org/10.1016/j.lwt.2004.lwt.2004.09.014.

Rojas-Graü, M. A., Raybaudi-Massilia, R. M., Soliva-Fortuny, R. C., Avena-Bustillos, R. J., McHugh, T. H., & Martín-Belloso, O. (2007). Apple puree-alginate edible coating as carrier of antimicrobial agents to prolong shelf-life of fresh-cut apples. *Postharvest Biology and Technology*, *45*, 254−264. http://dx.doi.org/10.1016/j.postharvbio.2007.01.017.

Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2009). Edible coatings to incorporate active ingredients to fresh-cut fruits: a review. *Trends in Food Science & Technology*, *20*, 438−447. http://dx.doi.org/10.1016/j.tifs.2009.05.002.

Vargas, M., Pastor, C., Chiralt, A., McClements, D. J., & González-Martínez, C. (2008). Recent advances in edible coatings for fresh and minimally processed fruits. *Critical Reviews in Food Science and Nutrition*, *48*, 496−511. http://dx.doi.org/10.1080/10408390701537344.

Wang, C. Y., Wang, S. Y., & Chen, C. (2008). Increasing antioxidant activity and reducing decay of blueberries by essential oils. *Journal of Agricultural and Food Chemistry*, *56*, 3587−3592. http://dx.doi.org/10.1021/jf7037696.

Zivanovic, S., Chi, S., & Draughon, A. F. (2005). Antimicrobial activity of chitosan films enriched with essential oils. *Journal of Food Science*, *70*, 45−51. http://dx.doi.org/10.1111/j.1365-2621.2005.tb09045.x.

Using natural antimicrobials to enhance the safety and quality of milk

15

M.C. Pina-Pérez, D. Rodrigo, A. Martinez
Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain

15.1 Introduction

Consumption of beverages in Europe during 2010 amounted to 210,264 million litres, including fruit juices (12,204 million litres), hot drinks including tea and coffee (87,257 million litres) and soy beverages (588 million litres) (Zenith, 2010). The milk-based product industry now covers a high percentage of the most-consumed novel and functional beverages, such as soy milk-based beverages, milk blended with vegetable beverages, fruit juices and liquid egg-based beverages. The beverage industry is becoming more attractive for consumers mainly because of the (1) functional properties, (2) a-caloric characteristics and (3) innovative tasting and sensory qualities of these novel products. Moreover, the scientific community and food processors are focusing on finding alternative technologies to enhance beverage quality and safety (Gruenwald, 2009; Keenan,Brunton, Mitchell, Gormley, & Butler, 2012). With these technologies, the beverage industry is becoming the fastest-growing segment of food products, providing consumers with high-quality active vegetable ingredients with valuable antioxidant, anti-ageing, anti-inflammatory, immune and antimicrobial properties.

Bioactive phytochemicals in plants are recognized as being responsible for the antimicrobial nature of these products, with additional health properties (Negi, 2012). New 'green additives' are well accepted by concerned consumers who reject the addition of chemicals to food products. Vegetables, fruits other plant matrices and microbial metabolites are generally recognized as rich sources of bioactive compounds and natural antimicrobials (Kim, Yang, Lee, & Kang, 2011). In the case of bioactive compounds, resveratrol added to juices and prebiotics in milk are examples of natural additives that contribute to the healthiness and quality of innovative beverages (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Prado, Parada, Pandey, & Soccol, 2008).

Not only the enhancement of beverage functionality but also the accomplishment of food safety can result from supplementation of beverages with natural compounds. In fact, natural antimicrobials can inhibit or inactivate spoilage microorganisms responsible for deterioration of food quality (flavour, aroma, colour, texture) and/or can inhibit/delay or inactivate pathogen growth, guaranteeing the food safety of beverages (Pina-Pérez, Martinez, & Rodrigo, 2012; Pina-Pérez, Silva-Angulo, & Rodrigo, 2012).

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At the same time, these natural antimicrobials can positively affect quality characteristics related to food products, including those directly affecting consumer choices (concerning appearance, colour, aroma, taste, freshness and, in general, organoleptically assessed attributes), as well as internal product characteristics not directly detected by consumers (health-promoting compounds, e.g. proteins, phenolic compounds).

Natural compounds with nutritional and antimicrobial properties are included in the field of non-thermal preservation hurdles applied to perishable foods, among them beverages, resulting in improved quality (flavour, texture, taste), extension of shelf life and reduced levels of microbial contamination and spoilage, and providing newly formulated products with functional properties. The study of the effect of these natural compounds on beverage quality and safety is the main area on which the present chapter focuses, addressing concerns such as (1) how the safety and quality of beverages can be enhanced by means of natural antimicrobials, (2) the effectiveness of these interventions and (3) the factors upon which these interventions depend. This chapter reviews examples of the most important reports that have appeared recently in the scientific literature about the effect of adding antimicrobial compounds obtained from herbs and spices, fruits and vegetables, leaves and bark, animal tissues and microorganisms, which can act alone or by interaction with other non-thermal technologies under the hurdle technology concept.

15.2 Enhancing the safety and quality of milk-based beverages using natural antimicrobials: milk

Although thermal processing is still the technology most commonly used for inactivating microorganisms and enzymes in general processed food products, and specifically beverages, it is well known that sensory and nutritional compounds are modified as a result. In this respect, the dairy industry is one of the most advanced food sectors regarding the application of novel technologies (Komitopoulou, 2013) to process milk-based products. In the area of natural bioactive compounds, essential oils, probiotics and prebiotics, and fatty acids, are the health-promoting compounds most commonly added to milk-based beverages that are also being studied to enhance product quality and safety by means of their functional and antimicrobial properties.

In addition, the application of non-thermal processes to ensure microbial inactivation of milk products with reduced effects on nutritional and quality parameters is under study, especially pulsed electric fields (PEFs) and high hydrostatic pressure (HHP), combined with the addition of natural bioactive compounds of animal (fatty acids), microbial (probiotics and prebiotics) and vegetable origin (essential oils, isoflavones) (Chipurura & Muchuweti, 2010).

With regard to milk products, the microbiological preservation of newly formulated milk-based beverages has been extensively studied by Martinez, Rodrigo and co-workers for some years (2006–2013) (Criado, Martínez, & Rodrigo, 2012; Pina-Pérez, Martinez, et al., 2012; Pina-Pérez, Rodrigo, & Martinez, 2009; Pina-Pérez,

Rodrigo, & Martinez, 2013; Pina-Pérez, Silva-Angulo, et al., 2012), by means of supplementation with natural antimicrobials, alone or in combination with other non-thermal technologies. At the same time, other research groups have also focused on this task because of the high commercial impact of dairy beverages and good acceptability of these products for consumers (Giroux, Acteau, Sabik, & Britten, 2008; Pérez-Pullido, Toledo del Árbol, Grande Burgos, & Gálvez, 2012; Élez-Martinez, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2012).

15.2.1 Antimicrobials of vegetable origin

Isoflavones. Soy has shown health benefits such as reduction of cardiovascular diseases, menopausal symptoms, osteoporosis and breast and prostate cancers. Specifically, isoflavonoid phenolic compounds have demonstrated valuable pharmacological and antimicrobial applicability (Devi et al., 2009). Dastidar et al. (2004) studied the effect of isoflavones from *Leguminosae* of the pea plant family against *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Pseudomonas* spp., *Vibrio cholerae* and *Vibrio parahaemolyticus* in milk. Isoflavones proved most active against *S. aureus*, and then, in decreasing order, against *V. cholerae*, *Salmonella*, *Shigella*, *Klebsiella* and *Pseudomonas* spp., with the minimum inhibitory concentration ranging from 25 to 200 mg/l for most microorganisms. *In vivo*, it was found that at concentrations of 30 and 60 g/mouse these compounds offered significant protection to mice challenged with a median lethal dose (MLD) of a virulent strain of *Salmonella* Typhimurium.

In spite of the antimicrobial potential of isoflavones in enriched soy-milk products, quality aspects should be improved because the main thermal treatments applied can affect the mouth feel and flavour of these beverages. Alternative processing technologies are under study to improve the quality and shelf life of these soy-milk beverages. According to Morales de la Peña, Salvia-Trujillo, Rojas-Graü, and Martín-Belloso (2010), high-intensity PEFs represent a technology that can potentially be applied as a pasteurization alternative to thermal treatments, maintaining the isoflavone content of a new soy-milk beverage treated at 35 kV/cm, 800−1400 μs. Even during storage at 4 °C, genistein, daidzein and daidzin isoflavone content increased. Moreover, after 56 days of refrigerated storage at 4 °C, the soy milk-based beverage did not differ significantly from the fresh product with regard to their high content of isoflavones and fresh-like characteristics.

Essential oils. In the dairy product context, various authors have pointed out the positive impact that essential oils (EOs) from plants could have on the safety and quality of milk-based beverages. Essential oils are aromatic and volatile liquids extracted from plants, secondary metabolites, which play an important role in plant defence because of their antimicrobial properties. Essential oils are the most studied natural antimicrobial compounds in plant extracts (Ayala-Zavala & González-Aguilar, 2011). Among them, oils obtained from citrus have been extensively produced and applied at the industrial level in concentrations ranging from 0.1 to 10 μl/ml (Ayala-Zavala & González-Aguilar, 2011). Essential oils from orange, lemon and grapefruit juice were specifically effective against *Salmonella enterica* serovar Typhimurium in milk (Bajpai, Back, & Kang, 2012). Among the EOs studied in

citrus, terpineol was the most effective, reducing *Salmonella* spp. contamination by 7, 4 and 3 $\log_{10}$-cycles in skimmed milk, low butterfat milk and whole milk, respectively.

Cinnamon, added to milk-based beverages alone or as an additional hurdle in combination with other new preservation technologies, is another of the EOs most commonly studied (Cava, Nowak, Taboada, & Marin-Iniesta, 2007; Pina-Pérez, Martinez, et al., 2012; Pina-Pérez, Silva-Angulo, et al., 2012). Cava et al. (2007) assessed the antimicrobial potential of cinnamon bark oil, cinnamon leaf and clove EOs added to milk and skimmed milk against *Listeria monocytogenes* at incubation temperatures of 7 and 35 °C. The results obtained confirmed that 1%, 10.5% and 11% (w/v) concentrations, respectively, were required to be bactericidal against this microorganism, and the bactericidal effect was not influenced by incubation temperature. However, the fat content showed a protective effect against the action of the EOs studied. In addition to the antimicrobial effect of EOs against food-borne pathogens, spoilage microorganisms have been characterized as being more sensitive to EO exposure. In fact, it is well known that 60% of EOs show inhibitory effects against yeasts and fungi, while 40% of them show some bactericidal effect against bacteria (Burt, 2004). Tserennadmid et al. (2011) studied the antimicrobial capacity of four essential oils obtained from clary sage, juniper, lemon and marjoram against wild-type isolates of the food-related yeasts *Geotrichum candidum*, *Pichia anomala*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* suspended in milk substrate. According to the results obtained, the lag phases were lengthened by increasing EO concentrations, while a significant reduction of growth rates was obtained only at the highest EO concentration studied (0.125 μl/ml). Lemon essential oil was the most effective against *Geotrichum candidum* in milk. In fact, pasteurized milk shelf life under refrigerated storage at 4 °C was increased to 56 days by the addition of orange EO to milk substrate.

Additive and synergistic effects can occur by interaction of EOs (García-García, López-Malo, & Palou, 2011). For example, Tserennadmid et al. (2011) found in milk that a combination of juniper and clary sage had an additive effect against *G. candidum*; and α-pinene and limonene had a synergistic effect on the substrate studied.

Not only antimicrobial but also antioxidant properties have been attributed to essential oils, improving milk product quality. The research work carried out by Boroski et al. (2012) focused on testing the efficacy of oregano extract (OE) (*Origanum vulgare*) and essential oil of oregano (OEO) (*Origanum minutiflorum*) (0.001−0.1 g/100 g) to increase the antioxidant capacity of pasteurized dairy beverages (63.5 °C, 30 min) enriched with 2 g/100 g of linseed oil. During 10 days of storage under light or heat conditions, colour, conjugated diene hydroperoxides, hexanal, propanal and dissolved oxygen concentrations were evaluated in dairy beverages containing various concentrations of OE or OEO. According to the results obtained, oregano extract exerted higher antioxidant activity measured as free-radical-scavenging effect than the essential oil extract, which can be explained by antioxidant chemistry. The polar characteristic of the phenolic compounds present in OE allows them to act better as antioxidants in the aqueous phase of the emulsion in comparison with OEO

compounds. The main compounds responsible for the antioxidant activity of OE are rosmarinic acid, caffeic acid, coumaric acid, quercetin and carvacrol, which have also been associated with antimicrobial properties against a wide variety of food pathogenic and spoilage microorganisms (Gutiérrez, Barry-Ryan, & Bourke, 2009; Lucera, Costa, Conte, & Del Nobile, 2012). The addition of OE or OEO did not affect dairy beverage physical stability during storage (Giroux et al. 2008; Jacobsen et al., 2008).

Phytosterols. Beverage functionality can also be improved by means of phytosterol (PS) supplementation. Plants are also sources of phytosterols and phytostanols, which may help reduce low-density lipoprotein (LDL) cholesterol levels. Among beverages, milk-based products are one of the sectors most commonly supplemented in this way. According to Alemany-Costa et al. (2012), two different plant sterols from tall oil and vegetable oil were incorporated in milk-based beverages, helping to achieve the daily recommended intake of 1 g in vegans. The results revealed that a PS profile was predominant in β-sitosterol, achieving stability after laboratory-scale processing of the beverages, with consequent promising future applications. Regarding phytosterols content, aloe vera is another ingredient increasingly used in beverage applications, especially dairy products. It contains more than 200 active substances, among them phytosterols with anti-inflammatory, antioxidant and antimicrobial properties (Tanaka et al., 2006).

Ginseng. Several studies have also attributed antimicrobial capability against various Gram-positive and Gram-negative pathogens and fungi to ginseng root and leaf extracts (Lee et al., 2012; Ludwickzuk, Wolski, & Holderna-Kedzia, 2006), the ginsenosides and phenolic compounds (flavonoids, phenolic acids and tannins) being responsible for this antimicrobial potential. In addition to the antimicrobial potential of ginsenosides, the incorporation of ginseng extracts to beverages has been found to contribute to improvement of cognitive functions in the elderly, which represents another novel and interesting approach in dairy beverages (Tárrega et al. 2012).

15.2.2 Probiotics and prebiotics

The addition of microorganisms and/or their metabolites to milk-based products in order to improve beverage quality, produce health benefits, extend milk-based product shelf life and/or improve their safety is a current practice and well accepted. Examples of this can be found in the increasing market for yogurt- and milk-based beverages supplemented with prebiotics and probiotics. Probiotics are another group of natural substances that can be added to milk beverages and that have functional and antimicrobial properties against food-borne pathogens. For example, Macedo (1997) developed a probiotic beverage with a mix of 35% buffalo cheese whey, 30% soy milk and 35% cow milk fermented by a mixed culture of *Lactobacillus casei* Shirota and *Bifidobacterium adolescentis*. A fermentative proportion of 1:5 between the lactic acid and bifidobacteria in a 5% inoculum guaranteed the preservation of the chemical, microbiological and sensorial stability of the fermented beverage for 28 days at 4 °C, ensuring good acceptability of the product throughout the storage period. With regard to the food quality of milk, bifidobacteria are

frequently added to dairy products (milk, soy milk, soybean milk, fermented soybean milk and fruit-flavoured dairy beverages), enhancing their usefulness to human health. In addition to the functional effects attributed to bifidobacteria, it has been reported that there is an antagonistic effect between *Bifidobacterium* proliferation and the growth of psychrotrophic microorganisms (*Bacillus cereus*, *Pseudomonas*, *Yersinia* and *Listeria*), preventing significant spoilage and public health threats. As antimicrobials, bacteriocins from lactic acid bacteria have shown effectiveness against *Listeria* spp. in milk, even at refrigeration abuse temperatures (15 °C). At refrigeration temperatures, below 10 °C, the observed effectiveness of bacteriocins increases, with a positive impact resulting from their addition to milk as preservatives in the chill chain. A delayed growth pattern was observed in *Listeria* spp. cells exposed to bacteriocins, with up to 30 days being required to reach the final count of 8 $\log_{10}$ cycles. This result helps to improve the safety of refrigerated milk products by extending their shelf life in comparison with the non-supplemented beverage, in which the final count achieved was 8 log cycles after 4 days at 15 °C (Vaughan et al., 1994). Moreover, combinations of bacteriocins have shown a more intensive effect than single bacteriocins, e.g. against *Listeria* spp. (Khan, Flint, & Yua, 2010), reducing bacterial counts to undetectable levels even after 30 days at 15 °C.

According to Servili et al. (2011), a novel research field has been opened around the combination of biogenic compounds from natural sources and functional lactic acid bacteria to develop milk-based beverages with enhanced quality, functional and possible antimicrobial properties. According to the results of Servili et al. (2011), phenolic compounds, extracted from olive vegetation water, at concentrations of 100 or 200 mg/l, and fermented with γ-amino butyric acid (GABA)-producing (*Lactobacillus* plantarum C48) and autochthonous human gastro-intestinal (*Lactobacillus* paracasei 15N) LAB, were suitable for new functional milk beverage enrichment with subsequent quality enhancement (increase in free amino acids during beverage fermentation) and good sensory acceptability by consumers. Moreover, olive vegetation phenolic enrichment of milk beverages (1 g/100 ml) contributes to optimal intake of phenols from Mediterranean virgin olive oil.

15.2.3 Fatty acids

Fatty acids, naturally occurring omega-3 polyunsaturated fatty acids from fish, algae and linseed oils, are particularly interesting for supplementation of milk-based beverages to achieve the daily intake of these substances that is recommended in order to obtain health benefits (Kris-Etherton, Harris, & Appel, 2002). Polyunsaturated fatty acids of the n-3 family (n-3 PUFA) have been associated with a reduced risk of coronary heart disease and prevention of epilepsy and prostate, colorectal and breast cancer. However, these oils are very susceptible to oxidative degradation (Giroux et al., 2008; Jacobsen et al., 2008). According to Giroux, Houde, and Britten (2010), the addition of pre-heated milk-derived protein−sugar blends to dairy beverages provides Maillard reaction products in the early stage of sterilization, which efficiently prevent lipid oxidation of dairy beverages. The use

of monosaccharide in the formulation of pre-heated milk protein−sugar blends greatly improved their antioxidant properties. Therefore, the quality of beverages fortified with omega-3 polyunsaturated fatty acids could be improved by avoiding oxidation during the sterilization process by adding blends of animal or vegetable fatty acids. Among the various potent biological activities of free fatty acids (FFAs) is the ability to kill or inhibit the growth of bacteria (Debois & Smith, 2010). Although their antibacterial mode of action is still poorly understood, the prime target of FFA action is the cell membrane, where FFAs disrupt the electron transport chain and oxidative phosphorylation. The antibacterial activity of each FFA is influenced by its structure and shape, medium- and long-chain unsaturated FFAs being more active against Gram-positive bacteria than against Gram-negatives. Fatty acid content has also been associated with antimicrobial properties of EOs, i.e. determining the antimicrobial potential of EOs, based on the percentage of fatty acids present in their chemical composition (Bajpai et al., 2012).

15.2.4 Hurdle technology applied to milk beverages

Hurdle technology, which involves multiple simultaneous preservation approaches, has generally met with success in controlling pathogens and maintaining food quality during storage (Leistner, 2000). In the area of guaranteeing milk beverage safety and quality, various authors have worked intensively to obtain minimally processed products with high nutritional and quality attributes, improving the organoleptic and nutritional value of thermally treated beverages and preserving the microbiological stability of pasteurized milk-based beverages during refrigerated shelf life. In this regard, non-thermal technologies such as PEFs and HHP have been studied in combination with natural antimicrobials to enhance microbial inactivation levels. Pina-Pérez, Martinez, et al. (2012) studied the combined effect of PEFs and cinnamon, added as an ingredient to a milk beverage in order to achieve suitable reduction levels while avoiding impairing the quality of the pasteurized milk. According to the results obtained by Pina-Pérez, Martinez, et al. (2012), at all the treatment conditions studied (electric field (E) 15−35 kV/cm; treatment time 60−3000 μs), a synergistic additional effect close to 1 $\log_{10}$ cycle was observed, attributed to a combination of non-thermal processing of skim milk and direct supplementation with 1%, 2.5% and 5% (w/v) of cinnamon powder.

Combined studies of HHP processing and addition of antimicrobials to milk substrate have shown additive and synergistic effects against Gram-positive and Gram-negative bacteria (Nakimbugwe, Masschalck, Anim, & Michiels, 2006; Nakimbugwe, Masschalck, Atanassova, Zewdie-Bosüner, & Michiels, 2006; Pina-Pérez, Rodrigo, et al. 2009). Nakimbugwe, Masschalck, Anim, et al. (2006) and Nakimbugwe, Masschalck, Atanassova, et al. (2006) studied the effect of hen egg white lysozyme (HEWL) and bacteriophage lambda lysozyme (LaL) in combination with high-pressure treatment on inactivation of *Escherichia coli* O157:H7, *Shigella flexneri*, *Yersinia enterocolitica* and *Salmonella* Typhimurium. LaL addition to milk substrate (224 U/ml) increased HHP inactivation levels for all the microorganisms studied from 0.4 $\log_{10}$ units to 2.7 $\log_{10}$ units.

15.3 Enhancing the safety and quality of infant milk formulas using natural antimicrobials

15.3.1 Antimicrobials of vegetable origin

Several natural antimicrobials have been proved to have bactericidal action against the pathogen most concerned in reconstituted powdered infant formula milk, *Cronobacter sakazakii*. Caprylic acid (Jang & Rhee, 2008; Nair, Joy, & Venkitanarayanan, 2004), *trans*-cinnamaldehyde (Amalaradjou, Hoagland, & Venkitanarayanan, 2009), polyphenol-rich cocoa powder (Pina-Pérez, Rodrigo, & Martinez, 2011), carvacrol, thymol, eugenol, diacetyl, cinnamic acid (Lee & Jin, 2008) and water-soluble muscadine seed extracts (Kim et al., 2009) have been tested against *C. sakazakii* (Table 15.1). *C. sakazakii* exposure to different concentrations of natural vegetable antimicrobials added to milk formula was studied, and the potential of these antibacterial ingredients was assessed at different temperatures. Among the natural antimicrobials studied, caprylic acid (30 mM) and muscadine seed extracts (Kim et al., 2009) produced the most effective reduction of *C. sakazakii* contamination levels ($\approx$7 $\log_{10}$ cycles), under

Table 15.1 Natural antimicrobials added to infant milk formula to inactivate *Cronobacter sakazakii*

Antimicrobial substance	References	Addition	Inactivation results
Monocaprylin	Nair et al. (2004)	0.25−50 mM/4−37 °C	2−6 $\log_{10}$
Caprylic acid	Jang and Rhee (2009)	5, 10, 20, 30 mM/25 °C	7.7 $\log_{10}$
Trans-cinnamaldehyde	Amalaradjou et al. (2009)	0−0.5% (w/v)/4−23 °C	5−7 $\log_{10}$
Nisin	Al-Nabulsi et al. (2009)	1500 IU/ml/ 21−37 °C	4 $\log_{10}$
Carvacrol, thymol, eugenol, diacetyl, cinnamic acid	Lee and Jin (2008)	MICs: 1.25−5 mmol/l	Growth inhibition
Muscadine seed extracts	Kim et al. (2009)	Muscadine seed powder/ 30−60 min	>7 $\log_{10}$
Lactic acid	Al-Holy, Castro, and Al-Qadiri (2010)	0.2% (v/v)/2−6 h	3−7 $\log_{10}$
Sodium caseinate fermentate	Hayes et al. (2009)	3.3% (w/v)/60 min	6 $\log_{10}$

specific intervention conditions, at moderate temperatures (25−45 °C) and even at short exposure times (<1 h) (Jang & Rhee, 2009; Kim et al., 2009).

According to Pina-Pérez et al. (2011), polyphenol-rich cocoa powder (12% minimum polyphenol content, dry weight) reduced *Cronobacter sakazakii* growth in milk formulas with bacteriostatic results. Although no significant differences were observed in specific growth rate (μ_{max}) values at 25 °C between the non-supplemented milk formula and milk formula supplemented with polyphenol-rich cocoa powder (1, 2.5% and 5% (w/v)), lag phase duration (λ) was doubled by addition of cocoa at 5% (w/v). The contribution of this natural ingredient to the safety of milk-derived beverages is an innovative industrial application of cocoa against various microbial pathogens.

15.3.2 Probiotics and prebiotics

Bifidobacteria have also been incorporated in baby foods (O'Sullivan, 2004). As non-digestible food ingredients which feed probiotic bacteria in the digestive tract, prebiotics promote the development of healthy gut flora by allowing friendly bifidobacteria to flourish, which then promotes the production of short-chain fatty acids. It is thought that this is one of the factors that have traditionally given breastfed babies a softer, more optimal stool consistency and a much lower incidence of colicky and gassy symptoms. The feeding of a formula supplemented with *B. bifidum* and *Streptococcus thermophilus* substantially reduces the incidence of acute diarrheal disease in infants (Hoyos, 1999). Moreover, many infant formula manufacturers are now adding prebiotics to their formula in the form of oligosaccharides, mostly galacto-oligosaccharides, i.e. obtained from lactose, enhancing the functional value of infant feed. Bifidobacteria also produce antimicrobial peptides, e.g. the peptides produced by *Lactobacillus acidophilus* against *Cronobacter* spp. (Hayes et al., 2009), with enhanced microbiological safety of formula supplemented with this probiotic.

15.3.3 Fatty acids

Most infant formulas now contain long-chain fatty acids (LCPs), which are part of the omega-3 and omega-6 series. These LCPs, often from fish oils, have been reported to have an important impact on brain and visual development. In addition to the contribution of these LCPs to infant diet by means of milk formula supplementation, antiviral properties have been reported for free fatty acids and monoglycerides. According to Jang and Rhee (2009), a beneficial effect could result from addition of LCPs to infant milk formula because of their possible protective effect for premature infants against syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), *Haemophilus influenzae* and Group B *Streptococcus*.

15.3.4 Peptides

Milk proteins are the most important source of bioactive peptides, although plant proteins, especially soybean, also contain potential bioactive sequences. The antimicrobial

activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins such as lactoferrin, lactoperoxidase and lysozyme. Antibacterial peptides are recognized as an important component of innate immunity. It has long been recognized that breast-feeding of infants provides protection from a range of enteric and respiratory infections. One of the most potent antimicrobial peptides described so far corresponds to a fragment of the whey protein lactoferrin named lactoferricin (LF). Al-Nabulsi et al. (2009) studied the antimicrobial effect of lactoferrin to control *C. sakazakii* by the addition of this bioactive peptide to reconstituted powder infant formula milk. Samples in the presence of LF were incubated at 37, 21 or 10 °C. According to Al-Nabulsi et al. (2009), reduction levels of at least 5 $\log_{10}$ cycles were achieved in the presence of LF at ≥2.5 mg/ml (37 °C, ≥4 h), at ≥2.5 mg/ml (21 °C, ≥8 h) and at ≥10 mg/ml (10 °C, ≥48 h).

15.3.5 Hurdle technology applied to infant milk formulas

Non-thermal technologies have scarcely been applied to ensure *Cronobacter sakazakii* inactivation in infant milk formulas and at the same time improve retention of nutritional factors. Specifically, according to Pina-Pérez, Aliaga, Bernat, Enguidanos, and López (2007), Pina-Pérez, Rodrigo, Saucedo-Reyes, and Martinez (2007), Pina-Pérez, Silva-Angulo, Muguerza, Rodrigo, and Martinez (2009), Pina-Pérez et al. (2013), Arroyo, Cebrián, Pagán, and Condón (2010) and Arroyo, Somolinos, Cebrián, Pagán, and Condón (2010), high hydrostatic pressure, pulsed electric fields and ultrasounds (Arroyo, Cebrián, Pagán, & Condón, 2011) appear to be the best alternatives to thermal pasteurization of reconstituted infant milk powders, with possible future applicability in hospital and industrial settings. Among the non-thermal technologies studied, PEFs appear to be the most versatile one for application in hospital neonatal care units, where *C. sakazakii* outbreaks could have the worst consequences, including the death of children. The combination of natural antimicrobials with other technologies remains novel and not completely defined. To our knowledge, only Pina-Pérez et al. (2013) and Arroyo, Somolinos, et al. (2010) have added cocoa [1−5] % (w/v) and citral, respectively, to reconstituted formula processed by PEFs. Pina-Pérez et al. (2013) achieved a maximum inactivation level of 4.41 $\log_{10}$ cycles by a combination of hurdles (cocoa addition (5% (w/v), PEF application (15 kV/cm, 3000 μs) and 4 h post-treatment refrigeration at 8 °C), whereas Arroyo, Somolinos, et al. (2010) achieved a maximum *C. sakazakii* reduction level of 3 $\log_{10}$ cycles by PEFs (25 kV/cm, 100 pulses) combined with citral (200 μl/l). Both studies confirmed the synergistic effect obtained from the application of hurdle technology. The study conducted by Pina-Pérez et al. (2013) indicates the important influence of the addition of natural antimicrobials to milk formula, achieving the maximum inactivation level obtained to date derived from the application of PEFs to reconstituted formula (4.41 $\log_{10}$ cycles) (Pina-Pérez et al., 2013).

15.3.6 New ingredients in infant feeding: regulation

The main factor limiting infant formula milk supplementation with natural antimicrobials is the approval of the incorporation of these natural bioactive compounds in infant diets. With regard to the addition of ingredients to infant milk formulas, all

regulations in various countries are in agreement with the following basic principle: *the introduction of new ingredients to infant milk formulas must pose no or minimal risk to infants.* Although food products in the United States that are designed and marketed for infants are regulated under the Federal Food, Drug and Cosmetic (FD&C) Act of 1938 (21 U.S.C. §301), there are no specific European regulations concerning the addition of new ingredients to infant formulas (Regulation EC no. 258/97 (EC, 1997)). According to the commentary of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition's Committee on Nutrition on the nutritional and safety assessment of breast milk substitutes and other dairy products for infants, appropriate clinical studies of nutritional and safety assessment should be performed, particularly for components and combinations of components that have not previously been included in infant formulas and other dietary products for infants. Technological and compositional modifications to infant formulas should be assessed nutritionally. In the case of natural ingredients added to milk formulas in order to improve quality and microbiological safety, adverse immunological changes and allergic effects are the main issues under study.

15.4 Enhancing the safety and quality of egg–milk beverages using natural antimicrobials

Egg and egg-derived products are generally used by the food industry not only as ingredients, because of their nutritional value as sources of animal protein and minerals, but also as additives because of the technological properties attributed to eggs and required by food producers. Currently, as a result of the new diet habits of consumers and the requirements of specific population sectors, especially children and the elderly, food processors are introducing innovations in milk beverages. Children and the elderly are population subgroups that require high nutritional intakes (2100–2500 kcal, with proteins 30%, lipids 20% and carbohydrates 50%), which could be provided by diet supplementation with suitable ingredients. Liquid egg–milk-based beverages are an example of ready-to-eat products that can help to satisfy the requirements of the elderly and children in a healthy way. However, the main concerns about egg-based beverages are the high microbial contamination of the raw products, and the possible loss of physical/technological properties as a result of the intensive thermal treatments required to guarantee food safety.

15.4.1 Antimicrobials of vegetable origin

Vegetable antimicrobials are being added to egg-based beverages as potential natural preservatives (Mosqueda-Melgar, Raybaudi-Massilia, & Martin-Belloso, 2008a, 2008b). However, phytochemical metabolites and vegetable extracts are not the only ingredients that can usefully be added to beverages for food safety and quality enhancement purposes. Recent studies based on by-product disposal and the use of unprocessed herbs and vegetables (leaves, powder, seeds) as ingredients to be added

to beverages are attracting attention (Dominguez-Perles, Moreno, Carvajal, & Garcia-Viguera, 2011; González-Sarrías, Larrosa, García-Conesa, Tomás-Barberán, & Espín, 2013; Pina-Pérez, Silva-Angulo, et al. 2012). Natural ingredients such as anise, cinnamon, cocoa and vanilla have been under study by our research group to quantify their bacteriostatic or bactericidal potential when added directly to beverage formulations. Although this practice has some disadvantages (lack of homogeneity, standardization of unknown phytochemicals), there are also some noteworthy advantages resulting from the antimicrobial properties exerted by these ingredients: (1) they are available for consumers to purchase and incorporate in natural meals made in the home, (2) they are minimally processed at low cost to food processors who wish to incorporate them in product formulations and (3) they are generally recognized as safe (GRAS), with no legal limitations. The ingredients just mentioned were incorporated at 5% (w/v) in a milk (80% (w/v))—egg ((20% (w/v))-based beverage to assess their antimicrobial potential to inhibit *Bacillus cereus* growth at different temperatures (22, 10 and 5 °C). According to our results, cinnamon and cocoa were able to inhibit bacterial growth, achieving reductions of up to 2.5 and 3.8 log cycles, respectively, of potential vegetative cell growth in an egg-based beverage stored at 5 °C. The antibacterial activity of cinnamon and cocoa was temperature-dependent, with a maximum effect at refrigeration temperature.

15.4.2 Peptides: lysozyme

Among the natural antimicrobials used in egg-derived products, lysozyme, which is an inherent part of the composition of an egg, has proved to be bactericidal against Gram-positive bacteria, *Listeria* spp. and *Bacillus cereus* (Abdou, Higashiguchi, Aboueleinin, Kim, & Ibrahim, 2007; Yang, Qu, Wimbrow, Jiang, & Sun, 2007). Nakimbugwe, Masschalck, Anim, et al. (2006) and Nakimbugwe, Masschalck, Atanassova, et al. (2006) studied the antimicrobial potential of hen egg white lysozyme (HEWL) and goose egg white lysozyme (GEWL) against a wide range of Gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis*, *Listeria innocua*, *S. aureus* and *Micrococcus lysodeikticus*) and Gram-negative bacteria (*Y. enterocolitica*, *S. flexneri*, *E. coli* O157:H7, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium). Inactivation due to exposure of Gram-positive bacteria to lysozymes achieved maximum levels of up to 5.6 $\log_{10}$ cycles, but no Gram-negative bacteria showed sensitivity to exposure solely to lysozymes. Gram-positive bacteria are generally more susceptible because they have a much simpler cell wall, consisting of a large extent (90%) of peptidoglycan, while in Gram-negative bacteria the peptidoglycan concentration in the cell wall is much lower (5−10%) and it is protected by an outer layer of lipopolysaccharide, which prevents lysozyme from reaching the substrate.

15.4.3 Bacteriocins

Simple addition of natural antimicrobials to liquid egg beverages could enhance their quality, especially if these substances are used as hurdles to reduce thermal treatment intensity. According to Miller et al. (2010), addition of the bacteriocins nisin and Micocin X, obtained from Lactic Acid Bacteria, to liquid whole egg (LWE) produced

a significant extension of shelf life under refrigeration (4 °C). Addition of Micocin X to LWE at levels in the range 0.2−1.6 mg/ml reduced the proteolytic and lipolytic action of lactic acid bacteria, helping to enhance LWE beverage quality during the storage period. The addition of 500 mg/mL of Micocin X followed by a thermal treatment of 65 °C for 2.5 min led to an extension of shelf life to a minimum of 5 weeks under refrigerated storage, exceeding the shelf life of LWE not supplemented with bacteriocin (maximum of 3 weeks).

15.4.4 Hurdle technology applied to egg−milk-mixed beverages

In addition to individual incorporation of the ingredients studied to egg-based beverages as preservatives or hurdles acting alone, these vegetable products can be incorporated in mixed beverages, contributing to the effect of other processing technologies in order to control food-borne pathogens. Recent scientific studies have incorporated minimally processed egg−milk mixtures in the field of beverage innovations in response to consumer demand for fresh products, satisfying consumers' energy and nutritional requirements (Geveke, 2008; Góngora-Nieto, Pedrow, Swanson, & Barbosa-Cánovas, 2003; Monfort, Gayán, Raso, Condón, & Alvarez, 2010). However, minimally processed egg-derived products (thermal treatments: 64.4 °C, 2.5 min (Wood & Waites, 1988); PEF- and HHP-treated products still under study (Monfort et al. 2010; Pina-Pérez, Martinez, et al., 2012; Pina-Pérez, Silva-Angulo et al., 2012)) are susceptible to *Bacillus cereus* germination and vegetative cell growth to infective dose levels (10^3 CFU/g) (EFSA, 2005), with a consequent risk to human health. Recent studies by Pina-Pérez, Rodrigo, et al. (2009); Pina-Pérez, Martinez, et al. (2012); Pina-Pérez, Silva-Angulo, et al. (2012) confirmed the effectiveness of 5% (w/v) polyphenol-rich cocoa in order to control *Bacillus cereus* vegetative cells in a liquid whole egg and skim milk (LWE-SM) mixed beverage processed by PEFs. Cocoa is an ingredient favoured by food processors and consumers because of its flavouring (De Clercq et al., 2012), technological (Norton & Fryer, 2012) and health properties (Schinella et al., 2010). Cocoa is consumed all over the world, and its antioxidant potential in association with phenolic compounds, mainly procyanidins and flavan-3-ols, is well known. These compounds have been associated with the antimicrobial properties of cocoa (Bubonja-Sonje, Giacometti, & Abram, 2011). According to studies by Pina-Pérez, Rodrigo, et al. (2009); Pina-Pérez, Martinez, et al. (2012), a synergistic effect was observed between cocoa addition and the application of PEF technology, achieving a maximum inactivation level of 3.30 $\log_{10}$ cycles after a 40 kV/cm-360 μs PEF treatment in a beverage supplemented with 5% (w/v) cocoa. Even during refrigerated storage for 15 days at 5 °C, the bacterial population continued decreasing by up to 4 $\log_{10}$ cycles. According to Pina-Pérez, Rodrigo, et al. (2009), it seems that addition of cocoa sensitizes the bacterial cells to simultaneous PEF treatment, leading to synergistic inactivation results. In addition to the well-known functional properties of cocoa polyphenols as health-promoting agents against the development of coronary heart and cardiovascular disease, compounds present in cocoa might be responsible for antimicrobial properties that alter physiological cell activities (Dreosti, 2000).

High hydrostatic pressure has also been studied as an alternative to thermal processing of egg-based beverages. According to Pina-Pérez, Silva-Angulo, et al. (2009), vegetable ingredients, cinnamon, cocoa, anise and vanilla, were added directly (700 ppm) to an LWE-SM formulated beverage, resulting in a synergistic effect between HHP processing and the addition of cinnamon, cocoa or vanilla to the egg-based beverage. Cocoa was the most bactericidal ingredient, acting as a hurdle with 300 MPa-12 min, with a maximum inactivation level of 5.57 $\log_{10}$ cycles after HHP processing. The membrane destabilization due to the HHP effect facilitates increased penetration of the active cocoa compounds, which are jointly responsible for the synergistic antimicrobial effect observed. With regard to the physico-chemical properties of egg-based beverages, according to Hasnikova et al. (2009), colour, rheological, foaming and emulsifying properties of LWE remain stable after HHP processing at 300 MPa-200 s.

15.5 Conclusion and future trends

Whenever new beverages are developed, it is important to go through every change made in the recipe, packaging and preservation in order to consider the microbial risks. Possible new microbial health risks in beverage production may arise from the increasing importation of ingredients worldwide and from the use of low-acid juices as ingredients. Consequently, deeper studies on process design, optimizing food quality while also satisfying safety constraints, will be a driving force for further evaluation of novel processes and formulations.

Modern beverages typically contain various potential growth enhancers and inhibitors, and their microbial stability is difficult to predict. The future challenge in beverage production is to produce safe and acceptably stable products with minimal processing, by combining antimicrobial hurdles in order to enhance microbiological quality while maintaining maximum sensory and nutritional quality. Hurdle technology that exploits the synergistic effect of existing natural antimicrobials, together with substances generally regarded as safe and mild physical preservation treatments, is a potential approach for controlling harmful pathogens and spoilage in beverage matrices.

References

Abdou, A. M., Higashiguchi, S., Aboueleinin, A. M., Kim, M., & Ibrahim, H. R. (2007). Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against *Bacillus* species. *Food Control*, *18*(2), 173–178.

Al-Holy, M. A., Castro, L. F., & Al-Qadiri, H. M. (2010). Inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*) in infant formula using lactic acid, copper sulfate and monolaurin. *Letters in Applied Microbiology*, *50*(3), 246–251.

Al-Nabulsi, A., Osaili, T. M., Al-Holy, M. A., Shaker, R. R., Ayyash, M. M., Olaimat, A. N., et al. (2009). Influence of desiccation on the sensitivity of *Cronobacter* spp. to lactoferrin or nisin in broth and powdered infant formula. *International Journal of Food Microbiology*, *136*, 221–226.

Alemany-Costa, L., González-Larena, M., García-Llatas, G., Alegría, A., Barberá, R., Sánchez-Siles, L. M., et al. (2012). Sterol stability in functional fruit beverages enriched with different plant sterol sources. *Food Research International*, *48*(1), 265−270.

Amalaradjou, M. A. R., Hoagland, T. A., & Venkitanarayanan, K. (2009). Inactivation of *Enterobacter sakazakii* in reconstituted infant formula by *trans*-cinnamaldehyde. *International Journal of Food Microbiology*, *129*, 146−149.

Arroyo, C., Cebrián, G., Pagán, R., & Condón, S. (2010). Resistance of *Enterobacter sakazakii* to pulsed electric fields. *Innovative Food Science and Emerging Technologies*, *11*, 314−321.

Arroyo, C., Cebrián, G., Pagán, R., & Condón, S. (2011). Inactivation of *Cronobacter sakazakii* by ultrasonic waves under pressure in buffer and foods. *International Journal of Food Microbiology*, *144*, 446−454.

Arroyo, C., Somolinos, M., Cebrián, G., Condón, S., & Pagán, R. (2010). Pulsed electric fields caused sublethal injuries in the outer membrane of *Enterobacter sakazakii* facilitating the antimicrobial activity of citral. *Letters in Applied Microbiology*, *51*, 525−531.

Ayala-Zavala, J. F., & González-Aguilar, G. A. (2011). Use of additives to preserve the quality of fresh-cut fruits and vegetables. In O. Martín-Belloso, & R. Soliva-Fortuny (Eds.), *Advances in fresh-cut fruits and vegetables processing* (pp. 231−254). Boca Raton: FL CRC Press.

Bajpai, V. K., Back, K. H., & Kang, S. C. (2012). Control of *Salmonella* in foods by using essential oils: a review. *Food Research International*, *45*, 722−734.

Boroski, M., Giroux, H. J., Sabik, H., Petit, H. V., Visentainer, J. V., Matumoto-Pintro, P. T., et al. (2012). Use of oregano extract and oregano essential oil as antioxidants in functional dairy beverage formulations. *LWT − Food Science and Technology*, *47*(1), 167−174.

Bubonja-Sonje, M., Giacometti, J., & Abram, M. (2011). Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. *Food Chemistry*, *127*(4), 1821−1827.

Burt, S. A. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*, 223−253.

Cava, R., Nowak, E., Taboada, A., & Marin-Iniesta, F. (2007). Antimicrobial activity of clove and cinnamon essential oils against *Listeria monocytogenes* in pasteurized milk. *Journal of Food Protection*, *70*, 2757−2763.

Chipurura, B., & Muchuweti, M. (2010). Effect of irradiation and high pressure processing technologies on the bioactive compounds and antioxidant capacities of vegetables. *Asian Journal of Clinical Nutrition*, *2*, 190−199.

Criado, M. N., Martínez, A., & Rodrigo, D. (2012). Use of Weibull distribution model to study the inactivation kinetics of peroxidase and polyphenoloxidase from orange, mango and papaya in presence of *Stevia rebaudiana*. In *2nd Workshop on Food safety: technologies and innovations applied to food safety* (pp. 317−326), ISBN 978-84-615-9669-0.

Dastidar, S. G., Manna, A., Kumar, K. A., Mazumdar, K., Dutta, N. K., Chakrabarty, A. N., et al. (2004). Studies on the antibacterial potentiality of isoflavones. *International Journal of Antimicrobial Agents*, *23*, 99−102.

De Clercq, N., Moens, K., Depypere, F., Vila Ayala, J., Calliauw, G., De Greyt, W., et al. (2012). nfluence of cocoa butter refining on the quality of milk chocolate. *Journal of Food Engineering*, *111*(2), 412−419.

Debois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology*, *85*, 1629−1642.

Devi, M. K. A., Gondi, M., Sakthivelu, G., Giridhar, P., Rajasekaran, T., & Ravishankar, G. A. (2009). Functional attributes of soybean seeds and products, with reference to isoflavone content and antioxidant activity. *Food Chemistry*, *114*(3), 771−776.

Dominguez-Perles, R., Moreno, D. A., Carvajal, M., & Garcia-Viguera, C. (2011). Composition and antioxidant capacity of a novel beverage produced with green tea and minimally-processed byproducts of broccoli. *Innovative Food Science & Emerging Technologies*, *12*(3), 361−368.

Dreosti, I. E. (2000). Antioxidant poliphenols in tea, cocoa, and wine. *Nutrition*, *16*(7/8), 692−964.

EFSA. (2005). Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. "*Bacillus cereus* and other *Bacillus* spp. in foodstuffs". *EFSA Journal*, *175*, 1−48. Available at http://www.efsa.eu.int

Élez-Martínez, P., Sobrino-López, A., Soliva-Fortuny, R., & Martín-Belloso, O. (2012). Chapter 4-Pulsed electric field processing of fluid foods. *Novel Thermal and Non-thermal Technologies for Fluid Foods*, 63−108.

European Commission. (1997). Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. *Official Journal L*, *043*, 0001−0006, 14/02/1997.

Fernández-Mar, M. I., Mateos, R., García-Parrilla, M. C., Puertas, B., & Cantos-Villar, E. (2012). Bioactive compounds in wine: resveratrol, hydroxytyrosol and melatonin: a review. *Food Chemistry*, *130*(4), 797−813.

García-García, R., López-Malo, A., & Palou, E. (2011). Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. *Journal of Food Science*, *76*, M95−M100.

Geveke, D. J. (2008). UV inactivation of *E. coli* in Liquid egg white. *Food Biprocess and Technology*, *1*, 201−206.

Giroux, H. J., Acteau, G., Sabik, H., & Britten, M. (2008). Influence of dissolved gases and heat treatments on the oxidative degradation of polyunsaturated fatty acids enriched dairy beverage. *Journal of Agricultural and Food Chemistry*, *56*, 5710−5716.

Giroux, H. J., Houde, J., & Britten, M. (2010). Use of heated milk protein−sugar blends as antioxidant in dairy beverages enriched with linseed oil. *LWT − Food Science and Technology*, *43*(9), 1373−1378.

Góngora-Nieto, M. M., Pedrow, P. D., Swanson, B., & Barbosa-Cánovas, G. V. (2003). Energy analysis of liquid whole egg pasteurized by pulsed electric fields. *Journal of Food Engineering*, *57*, 209−216.

González-Sarrías, A., Larrosa, M., García-Conesa, M. T., Tomás-Barberán, F. A., & Espín, J. C. (2013). Nutraceuticals for older people: facts, fictions and gaps in knowledge. *Maturitas*, *75*, 313−334.

Gruenwald, J. (2009). Novel botanical ingredients for beverages. *Clinics in Dermatology*, *27*, 210−216.

Gutiérrez, J., Barry-Ryan, C., & Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interaction with food components. *Food Microbiology*, *26*, 142−150.

Hasnikova, N., Jirincova, L., Kikulova, M., Landfeld, A., Mabek, M., Houska, M., et al. (2009). The influence of high pressure and/or antimicrobials on some functional properties of liquid whole egg. *Czech Journal of Food Sciences*, *27*, 228−233.

Hayes, M., Barrett, E., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2009). Evaluation of an antimicrobial ingredient prepared from a *Lactobacillus acidophilus* casein fermentate against *Enterobacter sakazakii*. *Journal of Food Protection*, *72*, 340−346.

Hoyos, A. B. (1999). Reduced incidence of necrotising enterocolitis associated with enteral administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to neonates in an intensive care unit. *International Journal of Infectious Diseases*, *3*, 197−202.

Jacobsen, C., Let, M. B., Sørensen, A. D. M., Horn, A. F., Timm-Heinrich, M., & Nielsen, N. S. (2008). Applications of natural antioxidants in omega-3 enriched foods. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, *7*, 3288−3295.

Jang, H. I., & Rhee, M. S. (2008). Inactivation of foodborne pathogens in reconstituted infant formula by caprylic acid. In *Poster presentation, annual meeting of Korean society of food science and technology,* 18 to 20 June, Gwangju, Korea.

Jang, H. I., & Rhee, M. S. (2009). Inhibitory effect of caprylic acid and mild heat on *Cronobacter* spp. (*Enterobacter sakazakii*) in reconstituted infant formula and determination of injury by flow cytometry. *International Journal of Food Microbiology*, *133*, 113−120.

Keenan, D. F., Brunton, N. P., Mitchell, M., Gormley, R., & Butler, F. (2012). Flavour profiling of fresh and processed fruit smoothies by instrumental and sensory analysis. *Food Research International*, *45*(1), 17−25.

Khan, H., Flint, S., & Yua, P.-L. (2010). Enterocins in food preservation. *International Journal of Food Microbiology*, *141*, 1−10.

Kim, T. J., Silva, J. L., Weng, W. L., Chen, W. W., Corbitt, M., Jung, Y. S., et al. (2009). Inactivation of *Enterobacter sakazakii* by water-soluble muscadine seed extracts. *International Journal of Food Microbiology*, *129*, 295−299.

Kim, I. S., Yang, M., Lee, O. H., & Kang, S. N. (2011). The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. *LWT − Food Science and Technology*, *44*(5), 1328−1332.

Komitopoulou, E. (2013). An overview of novel processing technologies for the food Industry. *Food Safety Magazine,* Issue 3, 14 June 2013. Available at http://www.foodsafetymagazine.com/signature-series/an-overview-of-novel-processing-technologies-for-the-food-industry/.

Kris-Etherton, P. M., Harris, W. S., & Appel, L. J. (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, *106*, 2747−2757.

Lee, S. Y., & Jin, H. H. (2008). Inhibitory activity of natural antimicrobial compounds alone or in combination with nisin against Enterobacter sakazakii. *Letters in Applied Microbiology*, *47*, 315−321.

Lee, O. R., Kim, Y. J., Balusamy, S. R. D., Khorolragchaa, A., Sathiyaraj, G., Kim, M. K., et al. (2012). Expression of the ginseng PgPR10-1 in *Arabidopsis* confers resistance against fungal and bacterial infection. *Gene*, *506*(1), 85−92.

Leistner, L. (2000). Hurdle technology. In *Food engineering, encyclopedia of life support systems (EOLSS),* Vol. III (pp. 1−7). Available at: http://www.eolss.net/sample-chapters/c10/e5-10-04-12.pdf.

Lucera, A., Costa, C., Conte, A., & Del Nobile, M. A. (2012). Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*, *3*(287), 1−12.

Ludwiczuk, A., Wolski, T., & Holderna-Kedzia, E. (2006). Estimation of the chemical composition and antimicrobial and antioxidant activity of extracts received from leaves and roots of American ginseng (*Panax quinquefolium* L.). *Kelva Polontea*, *52*(4), 79−90.

Macedo, R. E. F. (1997). *Desenvolvimento de bebida láctea fermentada a base de extrato hidrossolúvel de soja e soro de leite de búfala por cultura mista de* Lactobacillus casei *Shirota e Bifidobacterium adolescentis*, 112 f. Dissertação (Mestrado em Tecnologia Química), Universidade Federal do Paraná, Curitiba, 1997.

Miller, P., Haveroen, M. E., Solichova, K., Merkl, R., McMullen, L. M., Mikova, K., et al. (2010). Shelf life extension of liquid whole eggs by heat and bacteriocin treatment. *Czech Journal of Food Sciences*, *28*, 280−289.

Monfort, S., Gayán, E., Raso, J., Condón, S., & Alvarez, I. (2010). Evaluation of pulsed electric fields technology for liquid whole egg pasteurization. *Food Microbiology*, *27*(7), 845−852.

Morales-de la Peña, M., Salvia-Trujillo, L., Rojas-Graü, M. A., & Martín-Belloso, O. (2010). Impact of high intensity pulsed electric field on antioxidant properties and quality parameters of a fruit juice-soymilk beverage in chilled storage. *LWT − Food Science and Technology*, *43*(6), 872−881.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martin-Belloso, O. (2008a). Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices. *Food Microbiology*, *25*, 479−491.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martín-Belloso, O. (2008b). Inactivation of Salmonella enterica ser. Enteritidis in tomato juice by combining of high-intensity pulsed electric fields with natural antimicrobials. *Journal of Food Science*, *23*, M47−M53.

Nair, M. K. M., Joy, J., & Venkitanarayanan, K. S. (2004). Inactivation of *Enterobacter sakazakii* in reconstituted infant formula by monocaprylin. *Journal of Food Protection*, *67*, 2815−2819.

Nakimbugwe, D., Masschalck, B., Anim, G., & Michiels, C. W. (2006). Inactivation of gram-negative bacteria in milk and banana juice by hen egg white and lambda lysozyme under high hydrostatic pressure. *International Journal of Food Microbiology*, *112*, 19−25.

Nakimbugwe, D., Masschalck, B., Atanassova, M., Zewdie-Bosüner, A., & Michiels, C. W. (2006). Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. *International Journal of Food Microbiology*, *108*, 355−363.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, *156*, 7−17.

Norton, J. E., & Fryer, P. J. (2012). Investigation of changes in formulation and processing parameters on the physical properties of cocoa butter emulsions. *Journal of Food Engineering*, *113*(2), 329−336.

O'Sullivan, D. J. (2004). *Genomic approaches for functional food cultures*. International Food Conference. Thinking Beyond Tomorrow (pp. 17−18). June. Dublin, Ireland: National University of Ireland.

Pérez Pulido, R., Toledo del Árbol, J., Grande Burgos, M. J., & Gálvez, A. (November 2012). Bactericidal effects of high hydrostatic pressure treatment singly or in combination with natural antimicrobials on *Staphylococcus aureus* in rice pudding. *Food Control*, *28*(1), 19−24.

Pina-Pérez, M. C., Aliaga, D. R., Bernat, C. F., Enguidanos, M. R., & López, A. M. (2007). Inactivation of *Enterobacter sakazakii* by pulsed electric field in buffered peptone water and infant formula milk. *International Dairy Journal*, *17*, 1441−1449.

Pina-Pérez, M. C., Martinez, A., & Rodrigo, D. (2012). Cinnamon antimicrobial effect against *Salmonella typhimurium* cells treated by pulsed electric field (PEF) in pasteurized skim milk beverage. *Food Research International*, *48*, 777−783.

Pina-Pérez, M. C., Rodrigo, D., & Martinez, A. (2009). Sub-lethal damage in *Cronobacter sakazakii* subsp. *sakazakii* cells after different pulsed electric field treatments in infant formula milk. *Food Control*, *20*, 1145−1150.

Pina-Pérez, M. C., Rodrigo, D., & Martinez, A. (2011). Bacteriostatic effect of cocoa powder rich in polyphenols to control *Cronobacter sakazakii* proliferation in infant milk formula. In *Science and technology against microbial pathogens. Research, development and evaluation. Proceedings of the international Conference on antimicrobial research (ICAR 2010).* Valladolid, Spain, November 2010 (pp. 85−88). World Scientific Publishing Co. Pte. Ltd. ISBN-13 978-981-42354-85-1.

Pina-Pérez, M. C., Rodrigo, D., & Martínez, A. (2013). Cocoa powder as a natural ingredient revealing an enhancing effect to inactivate *Cronobacter sakazakii* cells treated by pulsed electric field in infant milk formula. *Food Control*, *32*, 87−92.

Pina-Pérez, M. C., Rodrigo, D., Saucedo-Reyes, D., & Martinez, A. (2007). Pressure inactivation kinetics of *Enterobacter sakazakii* in infant formula milk. *Journal of Food Protection*, *70*(10), 2281−2289.

Pina-Pérez, M. C., Silva-Angulo, A. B., Muguerza, B., Rodrigo, D., & Martinez, A. (2009). Synergistic effect of high hydrostatic pressure and natural antimicrobials on inactivation kinetics of *Bacillus cereus* in a liquid whole egg and skim milk mixed beverage. *Foodborne Pathogens and Disease*, *6*(6), 649−656.

Pina-Pérez, M. C., Silva-Angulo, A. B., Rodrigo, D., & Martinez, A. (2012). A preliminary expossure assessment model for *Bacillus cereus* cells in a milk based beverage: evaluating high pressure processing and antimicrobial interventions. *Food Control*, *26*, 610−613.

Prado, F. C., Parada, J. L., Pandey, A., & Soccol, C. R. (2008). Trends in non-dairy probiotic beverages. *Food Research International*, *41*(2), 111−123.

Schinella, G., Mosca, S., Cienfuegos-Jovellanos, E., Pasamar, M. A., Muguerza, B., Ramón, D., et al. (2010). Antioxidant properties of polyphenol-rich cocoa products industrially processed. *Food Research International*, *43*, 1614−1623.

Servili, M., Rizzello, C. G., Taticchi, A., Esposto, S., Urbani, S., Mazzacane, F., et al. (2011). Functional milk beverage fortified with phenolic compounds extracted from olive vegetation wáter and fermented with functional lactic acid bacteria. *International Journal of Food Microbiology*, *147*, 45−52.

Tanaka, M., Misawa, E., Ito, Y., Habara, N., Nomaguchi, K., Yamada, M., et al. (2006). Identification of five phytosterols from aloe vera gel as anti-diabetic compounds. *Biological and Pharmaceutical Bulletin*, *29*, 1418−1422.

Tárrega, A., Salvador, A., Meyer, M., Feullière, N., Ibarra, A., Roller, M., et al. (2012). Active compounds and distintice sensory features provided by American ginseng (*Panax quinquefolius* L.) extract in a new functional milk beverage. *Journal of Dairy Science*, *95*, 4246−4255.

Tserennadmid, R., Takó, M., Galgóczy, L., Papp, T., Pesti, M., Vágvölgyi, C., et al. (2011). Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *International Journal of Food Microbiology*, *144*, 480−486.

Vaughan, E. E., Caplice, E., Looney, R., O'Rourke, N., Coveney, H., Daly, C., et al. (1994). Isolation from food sources, of lactic acid bacteria that produced antimicrobials. *Journal of Applied Bacteriology*, *76*, 118−123.

Wood, S. L., & Waites, W. M. (1988). Factors affecting the occurrence of *Bacillus cereus* in liquid whole egg. *Food Microbiology*, *5*, 103−107.

Yang, H., Qu, L., Wimbrow, A., Jiang, X., & Sun, Y. P. (2007). Enhancing antimicrobial activity of lysozyme against *Listeria monocytogenes* using immunonanoparticles. *Journal of Food Protection*, *70*(8), 1844−1849.

Zenith International database. (2010). *Consumption of non-alcoholic beverages in the EU*. Apendix 3, pp. 1−2.

Using natural antimicrobials to enhance the safety and quality of fruit- and vegetable-based beverages

16

M.C. Pina-Pérez, D. Rodrigo, A. Martinez
Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain

16.1 Introduction

Chapter 15 identified the growing interest of consumers in beverages with enhanced sensory and nutritional properties. Bioactive phytochemicals in plants are now recognized for both their antimicrobial and health properties (Negi, 2012). As noted earlier, new 'green additives' are well accepted by concerned consumers who reject the addition of chemicals to food products. Vegetables, fruits, other plant matrices and microbial metabolites are generally recognized as rich sources of bioactive compounds and natural antimicrobials (Kim, Yang, Lee, & Kang, 2011). Natural antimicrobials can inhibit or inactivate spoilage microorganisms responsible for deterioration of food quality (flavour, aroma, colour, texture) and/or can inhibit/delay or inactivate pathogen growth, guaranteeing the food safety of beverages (Pina-Pérez, Martinez, & Rodrigo, 2012; Pina-Pérez, Silva-Angulo, Rodrigo, & Martinez, 2012). At the same time, these natural antimicrobials can positively affect quality characteristics related to food products, including those directly affecting consumer choices (concerning appearance, colour, aroma, taste, freshness and, in general, organoleptically assessed attributes), as well as internal product characteristics not directly detected by consumers (health-promoting compounds, e.g. proteins, phenolic compounds).

The study of the effect of these natural compounds on beverage quality and safety is the main area on which the present chapter focuses, addressing concerns such as (1) how the safety and quality of beverages can be enhanced by means of natural antimicrobials, (2) the effectiveness of these interventions and (3) the factors upon which these interventions depend. This chapter reviews examples of the most important reports that have appeared recently in the scientific literature about the effect of adding antimicrobial compounds obtained from herbs and spices, fruits and vegetables, leaves and bark, animal tissues and microorganisms, which can act alone or by interaction with other non-thermal technologies under the hurdle technology concept.

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16.2 Enhancing the safety and quality of fruit- and vegetable-based beverages using natural antimicrobials

According to FAO/WHO recommendations, an intake of five portions of fruit and vegetables per day is beneficial in terms of public health (Bates, 2010). Some fruits (apple, grapefruit, pineapple, cranberry, pomegranate, red grape, blueberry, mango, strawberry, black currant, cherry, açaí, tomato, beetroot, carrot and orange) and vegetables (cauliflower, soy, broccoli, stevia, cinnamon, clove, onion) are under study as beverage or food ingredients (mixed beverages, ready-to-eat products) because they are rich sources of natural bioactive compounds with tested health properties (Moure et al., 2001) and defined bactericidal or bacteriostatic capabilities (Raybaudi-Massilia, Mosqueda-melgar, & Martin-Belloso, 2009).

Fruits and vegetables are rich in functional food components such as minerals, vitamins, dietary fibre and antioxidants and do not contain any dairy allergens that might prevent usage by certain segments of the population (Luckow & Delahunty, 2004). However, some microorganisms and enzymes of concern might be present, compromising quality and safety in fruit- and vegetable-based beverages. They include *Salmonella* spp. and *Escherichia coli* O157:H7, generally recognized to be pathogens of concern in citrus fruit juices; spoilage microorganisms such as *Saccharomyces cerevisiae* (Élez-Martínez, Escolà-Hernández, Soliva-Fortuny, & Martín-Belloso, 2004; Parish, 2007), *Lactobacillus plantarum* and *Alicyclobacillus acidoterrestris*; and degradative enzymes such as polyphenol oxidase (PPO) and pectin methylesterase (PME). Losses of quality and safety of fruit and vegetable beverages due to microbiological proliferation are important for two main reasons: (1) the possible risk for consumers resulting from production of toxins and proliferation of pathogens and (2) financial losses derived from microbial spoilage. In this context, food processors and the scientific community are interested in the development of fruit- and vegetable-based functional beverages that comply with food safety standards (5 $\log_{10}$ cycles reduction of food-borne pathogens), with minimal degradation of nutritional and organoleptic profiles. Consequently, for processing optimization purposes from a quality and safety point of view, in the last 20 years various non-thermal technologies have been applied in combination with the addition of natural compounds to food matrices, under the concept of hurdle technology, in order to reduce microbiological risks and decrease organoleptic losses derived from the application of intensive physical treatments or use of chemicals. This section provides an overview of the main fruit- and vegetable-based beverages studied in terms of food safety implementation and quality improvement by means of the addition of natural ingredients.

16.3 Melon and watermelon juices

Melon and watermelon juices are characterized by low acidity (5.2−6.7) and high water activity (0.97−0.99) values, which provide optimal conditions for microbial proliferation. Because these fruits grow on the ground, microbial contamination with

Salmonella spp., *E. coli* O157:H7 and *Listeria monocytogenes* could take place when the fruit is processed. Mosqueda-Melgar, Raybaudi-Massilia, and Martin-Belloso (2008a) assessed the application of pulsed electric field technology in combination with citric acid and cinnamon bark oil from the viewpoint of juice quality and microbial reduction. Reduction levels of 5 $\log_{10}$ cycles were obtained in *E. coli* O157:H7, *Salmonella* Enteritidis and *L. monocytogenes* after 35 kV/cm-1709 μs and 35 kV/cm-1682 μs (at 193 Hz and 4 μs pulse duration) in melon and watermelon juices, respectively, containing 2.0% and 1.5% of citric acid, respectively, or 0.2% of cinnamon bark oil. These treatments were also effective to extend shelf life to 91 days of storage at 5 °C, as a result of inactivation of mesophilic, psychrophilic and mould and yeast populations.

In melon juices (pH 5.45), according to Raybaudi-Massilia et al. (2009), the resistance of the same microorganisms, *S*. Enteritidis, *L. monocytogenes* and *E. coli* O157:H7, was evaluated by adding malic acid to the juices at different concentrations. A concentration of 2.5% (v/v) of malic acid was required to reduce microbial populations by up to 5 $\log_{10}$ cycles when the juices were incubated at 5 °C for 24 h.

In general, depression of the internal pH of the microbial cell by ionization of undissociated acid molecules, disruption of substrate transport by altering cell membrane permeability or reduction of proton motive force, and chelation of metal ions essential for microbial growth (Eswaranandam, Hettiarachchy, & Johnson, 2004) are the main mechanisms for the effect of organic acids against microorganisms. Variations in effectiveness among acids depend on their molecular structure, size and pK_a (Eswaranandam et al. 2004; Parish & Higgins, 1989). Moreover, it is well known that the effectiveness of organic acids as antimicrobial agents (bacteriostatic/bactericidal) is influenced by juice characteristics, acid concentration, microorganism type (Gram-positive or Gram-negative), acid tolerance and storage temperature.

In addition to the acids most frequently added to melon and watermelon juices, essential oils have also been studied to improve the microbiological safety of these substrates. Lemongrass, cinnamon and geraniol essential oils, added at concentrations of 5 μg/ml, 8 μg/ml and 10 μg/ml, respectively, were required to inactivate *Salmonella enterica* serovar Typhimurium completely (>8 $\log_{10}$ cycles) in melon juices (pH 5.91) (Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2006).

Studies have also been conducted on the application of hurdle combinations to watermelon juices to preserve microbiological safety and extend shelf life, e.g. thermal treatment and addition of antimicrobials (Fazeli, Amirmozafari, Golbooi nejad, & Jamalifar, 2007). According to Fazeli et al. (2007), after a pasteurization process of 63 °C, 30 min, watermelon juices were inoculated with four strains of lactobacilli (*Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Lactobacillus plantarum*) as probiotics. Among the most beneficial effects associated with consumption of probiotics are balance of the gut bacterial population, synthesis of vitamins such as riboflavin and stimulation of the immune system. Moreover, these probiotics have been associated with antimicrobial properties due to microflora competitiveness in beverages. Lactobacilli were capable of growing in watermelon juice and reached a cell density of 10^8 CFU/ml after 48 h of incubation at 37° C.

All the probioticated watermelon juices were able to reduce the number of *S*. Typhimurium significantly (5−6 log reduction) after 2 h, *L. casei* being the most potent.

16.4 Orange and orange-based juices

Orange and orange-based juices are attractive to consumers because they contain high quantities of antioxidants, vitamins (A, B_6, B_{12}, C), dietary fibre, folate and other healthy compounds, including amino acids and phenolic compounds (Roussos, 2011). Antioxidant and anti-carcinogenic activities have been associated with citrus phytochemicals. They include carotenoids, flavonoids (hesperidin and narirutin as flavanones), saponins, steroids, terpenoids, tannins and alkaloids present in orange and lemon peel. The addition of natural antimicrobials to citrus juices is an innovative approach that is being followed by various research groups (Barba, Carbonell-Capella, Esteve, & Frigola, 2012; Liang, Mittal, & Griffiths, 2002; Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2009). Among the most important natural antimicrobials added to orange juices, naturally occurring compounds of plant origin (extracts and essential oils, e.g. cinnamon, vanillin and citral) and of animal origin (lactoperoxidase system and lysozyme), as well as organic acids, have been studied as antimicrobials against a wide range of spoilage and food-borne pathogenic bacteria (Mosqueda-Melgar et al., 2008a).

Kumar, Narayani, Subanthini, and Jayakumar (2011) assessed citrus peel extracts from an antimicrobial point of view and characterized them as valuable sources of natural phytochemicals with a high antimicrobial potential, equivalent to antibiotics such as metacillin or penicillin. The effectiveness of lemon and orange peel extracts was demonstrated against five pathogenic bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli*, *Klebsiella pneumonia* and *Salmonella* Typhimurium in orange juice at concentration levels ranging from 50 to 6.25 mg/ml. Other extracts with valuable antioxidant potential, carrot extract and β-carotene, the main antioxidant of carrot extract, have recently been corroborated by the studies of Hayashi et al. (2012) as antimicrobials against *Salmonella* Enteritidis when added to orange juice.

With regard to essential oils, cinnamon bark oil added at 0.1% (v/v) to orange juices reduced *E. coli* O157:H7 counts (Mosqueda-Melgar et al., 2008a). Vanillin and citral have also been incorporated in orange juices to control food-borne pathogens. According to Ferrante, Guerrero, and Alzamora (2007), vanillin added to orange juices combined with mild heat (45 °C) reduced the *L. monocytogenes* load by up to 2−3 $\log_{10}$ cycles (0.10−0.15% (w/v); 30 min) or 4 $\log_{10}$ cycles (0.20% (w/v); 15 min). Vanillin (4-hydroxy-3-methoxybenzaldehyde) has shown antimicrobial properties against *L. monocytogenes*, the initial count being reduced by up to 4 $\log_{10}$ cycles after 15 min of exposure to 2000 ppm of vanillin. On the other hand, addition of citral (3,7-dimethyl-2,6-octadienal) at 100 ppm prior to ultrasonication of an orange juice beverage (15 min, 600 W, 20 kHz, 95.2 μm wave amplitude) showed antimicrobial capability against *L. monocytogenes*, reducing bacterial counts by up to 2 $\log_{10}$ cycles. Although the mechanisms of action attributed to essential oils (EOs) have scarcely been studied, according to Oussalah, Caillet, and Lacroix (2006), the cytoplasmic

membrane is involved in the toxic action of EOs, and a general alteration of bacterial membrane integrity and induced depletion of intracellular adenosine triphosphate (ATP) concentration have been observed. Because of the increase in hydrophobicity that occurs at low pH values (Burt, 2004), a higher antimicrobial effect of EOs has been detected in low-pH fruit juices, such as orange or grapefruit, than in pear or melon juices. Fourier transform infrared (FT-IR) spectroscopy is one of the most promising physical techniques that have recently contributed to obtaining knowledge about the targets of natural antimicrobials. This technology is a potential method and is useful for the classification and identification of bacteria providing information of biochemical components of the cell wall and cytoplasm, including proteins and peptides, polysaccharides, peptidoglycan (murein), nucleic acids and phospholipids. In recent years, FT-IR has been widely used to discriminate, classify and identify various microorganisms (Anicuta, Dobre, Stroescu, & Jipa, 2010; Davis & Mauer, 2010), as well as to identify the chemical structure of antimicrobial substances and possible interactions between phytochemicals and microbial components (Lu et al. 2011).

A new approach to using natural antimicrobials (e.g. polyphenols and EOs) in foods was proposed by Gaysinsky, Davidson, Bruce, and Weiss (2005) and Donsì, Annunziata, Sessa, and Ferrari (2011), who employed a nano-encapsulation system for the entrapment of mixed substances. Donsì et al. (2011) studied the antimicrobial activity of encapsulated terpenes and D-limonene against *S. cerevisiae*, *E. coli* and *Lactobacillus delbrueckii* and found not only a higher effect of the nano-encapsulated compounds as specific antimicrobials but also an enhancement of beverage quality as a result of better preservation of the sensory properties of the pear and orange juices studied.

Other natural antimicrobials, such as nisin, the lactoperoxidase system and organic acids can reduce microbial pathogens and spoilage risks in orange-derived beverages when acting alone or combined with other technologies. Nisin is one of the antimicrobials most commonly added to fruit juices. It has been added to orange-based beverages to test its possible antimicrobial potential against natural juice microflora and against food-borne pathogens (*Salmonella* Typhimurium) (Rupasinghe & Yu, 2012). The addition of nisin (0.1 μg/ml) to pasteurized freshly squeezed orange juice led to a reduction of 2.95 $\log_{10}$ cycles in the initial load of *Salmonella* Typhimurium when the juice was processed by non-thermal pulsed electric fields (PEF) technology (30 pulses of 90 kV/cm) (Liang et al., 2002). According to the results of Hodgins, Mittal, and Griffiths (2002), combined treatments of PEF (80 kV/cm), mild heat (44 °C) and addition of nisin (100 U/ml) to orange juice achieved natural microflora inactivation levels of up to 6 log cycles. More intensive treatments, 90 kV/cm, 100 μs, mild heat of 55 °C and addition of nisin (100 U/ml), were required to inactivate a minimum of 7 $\log_{10}$ cycles of *Salmonella* Typhimurium in orange juice matrices.

Lactoperoxidase, the hemoprotein present in milk, has been characterized as exerting a broad spectrum of antimicrobial effects against bacteria, fungi and viruses (Naidu, 2000). The lactoperoxidase system exerts its antimicrobial action through short-life oxidation products, mainly hypothiocyanate ($OSCN^-$) and hypothiocyanous acid (HOSCN), with microbiocidal or microbiostatic effects by damage to the outer membrane, cell wall or cytoplasmic membrane, transport systems, glycolytic

enzymes and nucleic acids (Touch, Hayakawa, Yamada, & Kaneko, 2004). The US Food and Drug Administration (FDA) reported (2006) that lactoperoxidase is generally recognized as safe (GRAS) for use as an ingredient in foods, including fruit and vegetable juices (up to 167 mg/l). According to Van Opstal, Bagamboula, Theys, Vanmuysen, and Michiels (2006), a lactoperoxidase system was effective in reducing the *E. coli* O157:H7 bacterial load by up to 2 to 5 $\log_{10}$ cycles in orange juice.

Ascorbic, citric and malic acids, benzoic acid, potassium sorbate and calcium lactate are some of the organic acids most commonly used to reduce spoilage and pathogenic bacteria (*E. coli* O157:H7 and *Salmonella* Typhimurium) in fruit juices (Raybaudi-Massilia et al., 2009). Orange juice spoilage bacteria and yeast cause physical damage and chemical changes, such as oxidation and colour changes, or the appearance of off-flavours and off-odours resulting from microbial growth and the associated metabolism of the product. Spoilage bacteria cause food to deteriorate and result in unpleasant odours, tastes and textures. With regard to the main spoilage bacteria in orange juices, *Saccharomyces* spp., *Lactobacillus* spp., *Erwinia* spp., *Enterobacter* spp., *Alicyclobacillus* spp., *Propionibacterium cyclohexanicum* and *Pseudomonas* spp. have been studied (Walker & Phillips, 2008). According to Walker and Phillips (2008), *Propionibacterium cyclohexanicum* and *Alicyclobacillus acidoterrestris* are reduced by more than 5 $\log_{10}$ cycles by the addition of potassium sorbate or sodium benzoate to orange juice at a concentration of 1% (w/v). Antibacterial and antioxidant properties of ascorbic and citric acids have been demonstrated in many other food systems (Mahrour, Caillet, Nketsia-Tabiri, & Lacroix, 2003). The mechanism of the inhibitory effect of these compounds is related to the undissociated form of the acids, which diffuse freely through the cell membrane (Mahrour et al., 2003).

From a quality point of view, according to Criado, Martínez, and Rodrigo (2012) the antioxidant potential of a new orange juice mixed beverage could be improved by the addition of a new vegetable ingredient, *Stevia rebaudiana* Bertoni. *S. rebaudiana* Bertoni is a perennial shrub that originated in tropical and subtropical regions of South America and that has been used as a natural sweetener for hundreds of years, with associated bioactive properties (Muanda, Soulimani, Diop, & Dicko, 2011). The dry extract from *Stevia* leaves also contains phenolic compounds that give the plant antioxidant and antimicrobial properties. *Stevia* was added to an orange mixed beverage as a natural sweetener and the antioxidant capability of this new ingredient was quantified by determining the polyphenol oxidase (PPO) and peroxidase (POD) inhibition rates. The results showed that the greater the *Stevia* concentration, the faster the enzyme inhibition, POD being completely inhibited in the presence of 2.5% (w/v) of *Stevia*. Criado et al. (2012) concluded that under these conditions *Stevia* could be used as an anti-browning agent.

With regard to the application of hurdle technology to orange-based juices, recent studies have focused on microbiological safety, preservation and quality improvement, using the most commonly studied novel non-thermal technologies in combination with natural antimicrobials. In order to reduce microbiological contamination, McNamee et al. (2010) incorporated nisin (2.5 ppm), natamycin (10 ppm), benzoic acid (BA; 100 ppm) or lactic acid (LA; 500 ppm) in orange juice processed by PEF technology in order to inactivate spoilage (*Pichia fermentans*) and pathogenic

(*E. coli* and *Listeria* spp.) microorganisms. Combinations of nisin and PEF (40 kV/cm, 100 μs) showed the most synergistic effect on inactivation levels, producing a reduction of up to 5 $\log_{10}$ cycles or more in all the microorganisms studied. The synergistic effects on bacterial inactivation levels obtained by combining addition of EOs with minimal processing of juices are also well known and have recently been confirmed by studies conducted by Espina et al. (2012) and Arroyo, Somolinos, Cebrián, Condón, and Pagán (2010). Combinations of heat (54 °C, 10 min) or PEF treatment (30 kV/cm, 25 pulses) with addition of limonene (+) (200 μl/l) to orange juice were studied by Espina et al. (2012), confirming that synergistic effects of four extra $\log_{10}$ cycles are achievable by application of this hurdle technology. Similarly, Arroyo et al. (2010) confirmed the synergistic effect of applying PEF and adding citral to an orange juice beverage against *Cronobacter sakazakii*, achieving a reduction of up to 2 extra $\log_{10}$ cycles in bacterial counts.

From a quality point of view, the heating techniques most frequently used can result in oxidation, degradation and leaching to the water in the juices. To solve this problem, specifically in added value juices from 'superfruits', studies have reported an advantage of hurdle combinations over thermal treatments. Barba et al. (2012) worked with an orange mixed juice beverage supplemented with a new sweetener ingredient, *S. rebaudiana* Bertoni. To enhance the quality of the newly formulated product, the beverage supplemented with 2.5% (w/v) of *Stevia* was processed by high hydrostatic pressure (HHP) (300−500 MPa, 15 min), and the impact of this hurdle combination on the antioxidant capacity of the new orange mixture was assessed. According to the results, HHP enhanced the antioxidant capacity of the beverage studied because of the effect of this technology on the release of bioactive compounds from the fruit and/or *S. rebaudiana* extracts.

16.5 Grape juices

Supplementation of grape juices has seldom been carried out so far, possibly because of the inherent antimicrobial and antioxidant nature of these juices. Among acidic juices, grape juice (pH 3.0−3.3) is remarkable for the phenolic compounds it contains − mainly catechin, epicatechin, rutin, myricetin, *trans*-resveratrol, gallic acid, caffeic acid, ferulic acid, ellagic acid and quercetin (Butkhup, Chowtivannakul, Gaensakoo, Prathepha, & Samappito, 2010; Dahan & Amidon, 2009). Grape juice has never been implicated in *Salmonella* Typhimurium and *E. coli* O157:H7 outbreaks. Moreover, the antibacterial activity of grape juice and seed extract of a *Vitis vinifera* variety against *L. monocytogenes* has been reported by Rhodes, Mitchell, Wilson, and Melton (2006). The other most important pathogenic Gram-positive bacterium in foods, *Bacillus cereus*, was reduced by grape juice by nearly 1 $\log_{10}$ cycle after 10 min of exposure to anthocyanins in the juice studied, but this reduction rate did not increase with exposure time (60 min of study).

Naturally occurring spoilage bacteria were reduced by the intervention of natural antimicrobials according to Wu, Mittal, and Griffiths (2005). Nisin (400 U/ml) and lysozyme (0.4 g/100 ml) were added independently and jointly to grape juices, and

in both cases a significant decrease in juice microflora was observed. The application of hurdle technology to these juices is being studied increasingly. The antimicrobial effect of combined processes, such as PEF (80 kV/cm, 20 pulses) and mild heat (51 °C), with nisin supplementation can increase the reduction in bacterial counts, up to a maximum of 6.2 $\log_{10}$ cycles reduction by the three preserving factors (PEF + mild heat + nisin) acting in combination.

Moreover, grape seeds and grape skin pomace have been studied for their potential as natural ingredients with antioxidant and antimicrobial properties (Azizah, Ruslawati, & Swee, 1999; Martinez-Teruel, Sánchez, Megías, & Barbera, 1998). According to Su and Souza (2011), many pharmacological properties can be attributed to grape seed extract (GSE). In fact, the infectivity of human enteric virus exposed to GSE (2 h, 37 °C) at concentrations of 0.5, 1 and 2 mg/ml was reduced by up to 4.61 $\log_{10}$ PFU/ml, indicating the promising potential of GSE application in the food industry as an inexpensive novel natural alternative in order to reduce viral contamination and enhance food safety.

16.6 Apple and pear juices

Apple juice is a mixture of sugars (primarily fructose, glucose and sucrose), oligosaccharides and polysaccharides (e.g. starch), together with malic, quinic and citramalic acids, tannins (i.e. polyphenols), amides and other nitrogenous compounds, soluble pectin, vitamin C, minerals and a diverse range of esters that give the juice a typical apple-like aroma (e.g. ethyl- and methyl-iso-valerate).

A great variety of studies have been conducted on the antimicrobial effect of plant extracts, essential oils and combinations of them with other preservation techniques, added to apple juice matrix. Essential oils from palmarosa whole plant (*Cymbopogon martinii*), cinnamon (*Cinnamomum zeylanicum*) and clove (*Eugenia caryophyllata*) leaves and benzaldehyde and geraniol were tested for their bactericidal effect against *E. coli*, *Salmonella* Enteritidis and *Listeria innocua* (Raybaudi-Massilia et al., 2006). Addition of cinnamon to apple juice showed a marked killing effect (5 $\log_{10}$ cycles) against *L. monocytogenes* (1 h, 5−20 °C, 0.1−0.3% cinnamon addition to apple juice, pH 3.7−5.0) (Yuste & Fung, 2002) and *E. coli* O157:H7 (2.5% and 5% (v/v) in apple juice, even after 7 days of incubation at 8 °C (Ceylan, Fung, & Sabah, 2004). *E. coli* O157:H7 is one of the microorganisms that causes most concern in fruit juices because of its resistance to heat and acidic conditions. Consequently, in 2001 the Food and Drug Administration's Guidance for Industry included a requirement that all juice producers follow an *E. coli* O157:H7 5-$\log_{10}$ reduction rule in their Hazard Analysis and Critical Control Point (HACCP) controls (FDA, 2001). Espina et al. (2012) combined orange, mandarin or lemon EOs with mild heat to determine the inactivation effect on *E. coli* O157:H7 populations. A synergistic effect was observed between addition of citrus EOs to apple juice and application of mild heat, achieving a maximum of 5 $\log_{10}$ cycles' reduction when 200 μl/l of lemon EO was added to the juice and combined with 54 °C, 10 min. Friedman, Henika, Levin, and Mandrell (2004) carried out a deeper study, characterizing the antimicrobial effectiveness of various plant essential

oils (*Melissa* oil, terpineol, linalool, carvacrol, oregano oil, geraniol, eugenol, cinnamon leaf oil, citral, clove bud oil, lemongrass oil, cinnamon bark oil and lemon oil) against *E. coli* O157:H7 and *S. enterica*. The conclusions drawn can be summarized as follows: (1) the antimicrobial capability of the extracts was not conditioned by juice acidity, (2) *Salmonella* was more sensitive than *E. coli* O157:H7 to the extracts studied and (3) the antimicrobial effect increased with incubation temperature and time. However, at refrigeration temperatures (4 °C), addition of carvacrol, cinnamaldehyde, citral and thyme oil to unpasteurized apple juice can inactivate both these pathogens, preventing human exposure to health risk situations. Moreover, carvacrol, cinnamaldehyde, geraniol, linalool and terpinen-4-ol killed the bacteria after 5 min, while most of the essential oils studied were able to inactivate the pathogens after 30 min. Maximum inactivation levels (8 $\log_{10}$ cycles) as a result of EO intervention were achieved in *E. coli*, *S.* Enteritidis and *L. innocua* populations by adding lemongrass, cinnamon or geraniol (2 μl/ml) to apple and pear juices (pH 4.20 and 3.97, respectively). Although the exact mechanism(s) or target(s) for food antimicrobials are often not known or well defined, the antimicrobial capability of EOs has been reported to be strongly linked to the chemical composition of the intervening molecules (e.g. phenolics containing OH− groups work effectively against food-borne pathogenic bacteria) (Burt, 2004). Generally, the active compounds of essential oils have been recognized to have a phenolic structure, and, although it is difficult to identify a specific action site because many interacting reactions take place simultaneously at different cell sites, these phenolic compounds can disrupt the cell membrane and also effectively inhibit the functional properties of the cell, and may occasionally cause loss of intracellular material.

With regard to combining non-thermal processes, Bevilacqua, Campaniello, Speranza, Sinigaglia, and Corbo (2012) used HHP to process an apple juice supplemented with natural antimicrobials (limonene (900 ppm) and citrus extract (2 ppm)) as hurdles against *Saccharomyces bayanus* growth. According to the results obtained, HHP homogenization treatment at 20 MPa reduced the colony count of *S. bayanus* by 2−4 $\log_{10}$ CFU/ml, with citrus extract supplementation proving more effective than limonene to control yeast growth for 4−8 days of storage at 25 °C. Another hurdle combination proved effective against *A. acidoterrestris* spores present in apple juices. According to Bevilacqua, Corbo, and Sinigaglia (2010), *A. acidoterrestris* spores were controlled by a combination of cinnamon and eugenol EOs. The application of 40 ppm cinnamaldehyde with 40 ppm of eugenol or 80 ppm eugenol alone preserved apple juice for 7 days. Furthermore, this combination of cinnamaldehyde and eugenol as an apple juice preservative against *A. acidoterrestris* showed acceptable results in test panels (Bevilacqua et al., 2010).

16.7 Dark fruit juices

This category includes juices such as cranberry and blueberry and other dark fruits, such as cherry and black currant, that contain high levels of polyphenolic compounds (e.g. flavanoids, flavonoids and tannins), with reported bioaccessible antioxidant and

antimicrobial properties (Mitic, Obradovic, Kostic, Naskovic, & Micic, 2011). According to Seeram et al. (2008), the term *superfruits*, based on studies of the phytochemical composition of blackberries, has been used to name these fruits because of their high antioxidant content (85−92 mol TE/g), which has been associated with health and anti-ageing properties (Ryan & Prescott, 2010; Wootton-Beard & Ryan, 2011). Specifically, this high antioxidant capacity and polyphenol content are mainly responsible for the antimicrobial potential of these fruits, and only small quantities of antimicrobials need to be added to preserve the quality and safety of these dark fruit-derived juices. Moreover, the addition of these dark fruit juices to other matrices is an increasingly popular approach that improves the functionality and antimicrobial properties of newly formulated products. Some examples of this have been indicated by Ingham, Schoeller, and Engel (2006) and Biswas, Balac, Narlakanti, Haque, and Hassan (2012) regarding the incorporation of cranberry and blueberry juices in mixed beverages.

Cranberry juice is a food product with an attractive red colour and recognized health properties, mainly attributed to anthocyanin pigments: galactosides and arabinosides of cyanidin and peonidin. These compounds and other phytochemicals found in cranberries (flavanols and benzoic and cinnamic acid derivatives) have also attracted a great deal of attention, mainly because of their antioxidant, antibacterial, anti-inflammatory and anti-mutagenic properties (Coté, Caillet, Dussault, Sylvain, & Lacroix, 2011; White, Howard, & Prior, 2010). Cranberry juice and cranberry extracts may thus be used as a natural solution in the food industry, creating an additional barrier to inhibit the growth of food-borne pathogens while providing additional health benefits (Vattem & Shetty, 2002). In this context, Ingham et al. (2006) supplemented unpasteurized apple cider with cranberry juice (15% (v/v)) to enhance the lethality of a new non-thermal treatment, warm hold (45 °C for 2 h) and freeze-thaw steps (−20 °C for 24 h, 5 °C for 24 h). The cran-cider process achieved a $\geq$ 5-log reduction in numbers of *E. coli* O157:H7, *Salmonella* serovars and *L. monocytogenes* due to the intervention of cranberry juice.

Similarly, according to Biswas et al. (2012), supplementation of a milk beverage with blueberry juice enhanced the lethality of the beverage against food-borne pathogens while preserving probiotic milk flora. Biswas et al. (2012) studied the antimicrobial effect of a probioticated (*Lactobacillus bulgaricus* and *Bifidobacterium bifidum*) milk beverage enriched with blueberry juice as an antimicrobial hurdle against *Salmonella* Typhimurium, *Campylobacter jejuni*, *L. monocytogenes* and enterohemorrhagic *E. coli* O157:H7. All four pathogens were reduced by 4−7 $\log_{10}$ cycles in the beverage studied, but probiotic bacteria were able to survive and grow. Blueberries are rich in a variety of anthocyanins, flavanoids and tannins, which, as in all dark fruits, have been associated with their antimicrobial potential, e.g. against *L. monocytogenes*, enterohaemorrhagic *Escherichia coli* (EHEC), *Salmonella* Typhimurium and *S. aureus* (Burdulis et al., 2009).

Among dark fruits, açaí berries have a high antioxidant potential (410.8 μmol ET/g), due specifically to the presence of anthocyanins (cyanidin, epicatechin and catechin) and flavonoids (Del Pozo-Insfran, Brenes, & Talcott, 2004; Neida & Elba, 2007; Schauss, 2010). The use of açaí pulp and juices as natural ingredients in the formulation of novel products is innovative because nowadays the consumption of this fruit is

basically in the form of juices, mixed juice beverages with guaraná, milk-based beverages, desserts and ice-creams (Neida & Elba, 2007). The health benefits and nutritious bioactive composition of these fruits have been studied in depth (Akpinar-Bayizit, Ozcan, & Yilmaz-Ersan, 2012; Haydock & Hageman, 2007, p. 1), but the antibacterial properties of this natural product remain unknown. According to the results obtained by Belda, Pina-Pérez, Jiménez, Martinez, and Rodrigo (2012), only a bacteriostatic effect was attributed to this ingredient when added to a simulated food matrix and maintained at abuse incubation temperatures (37, 22, 12 °C).

16.8 Tomato juices

Organic acids, essential oils (EOs) and compounds extracted from vegetable by-products are some of the main natural antimicrobials incorporated in tomato juices to preserve their microbiological safety and extend their shelf life. There is growing interest in vegetable by-product extracts because of their antioxidant and fibre-rich properties, which makes them potential ingredients to be studied in food science. Phenolic-enriched extracts from blanched artichoke, artichoke-blanching water, cauliflower, carrot, celery and onion were used by Larrosa, Llorach, Espin, and Tomás-Barberán (2002) to increase the antioxidant activity of tomato juice, increasing the quality and functionality of the beverage. According to the results obtained, the antioxidant activity of the tomato juice studied was increased up to 5.4-fold over the control by supplementing the juice with artichoke-blanching water.

Mixtures of potassium sorbate and sodium benzoate have been added to tomato juices as an additional antimicrobial barrier during pasteurized juice storage. By supplementing tomato juice with potassium sorbate/sodium benzoate, ascorbic acid, total amino acids, fructose, glucose, lycopene and β-carotene contents remained constant during juice shelf life, both at 5 and at 20 °C storage temperatures, and at the same time yeast and mould counts decreased (Bizri & Wahem, 2006). The lactoperoxidase system has been applied by various authors to reduce tomato juice microbial contamination (Touch et al., 2004; Van Opstal et al., 2006). Van Opstal et al. (2006) found a slight effect of lactoperoxidase (LPER) systems, reducing <1 log cycle of *Shigella* spp., while Touch et al. (2004) reduced more than 5 $\log_{10}$ CFU/ml of *S*. Enteritidis in tomato juice treated with 14.8 μg/ml of LPER after 3 h (acid-adapted cells) and 4 h (non-adapted cells) of storage at 30 °C.

Concentrations of 2.0% of citric acid or 0.1% of cinnamon bark oil were needed to inactivate *S*. Enteritidis by more than 5.0 $\log_{10}$ units (5.08 and 6.04 $\log_{10}$ reductions, respectively) when tomato juices were processed by PEF technology (35 kV/cm, 1000 μs) (Mosqueda-Melgar et al., 2008a; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2008b). Other successful hurdle combinations, such as mild heat (50 °C), PEF (20 pulses, 80 kV/cm) and nisin (100 U/ml), can reduce *Salmonella* contamination levels in tomato juice by up to 4.4 $\log_{10}$ cycles. The combination of mild temperatures, 44 and 50 °C, with mint, a natural ingredient (0.1−1.2%), proved effective in reducing *Salmonella* counts by 5 to 8 $\log_{10}$ cycles in tomato juice (Nguyen & Mittal, 2007).

16.9 Other vegetable beverages

Among vegetable juices, cabbage, celery, pumpkin, eggplant, lettuce, spinach, leek and green bean juices have been studied from a microbiological stability point view, with the addition of a natural antimicrobial. The antimicrobial activity of a bacteriocin from *Enterococcus*, enterocin AS-48 (70 AU/ml), was tested in these vegetable juices, and significant antimicrobial activity was obtained in cabbage juice, followed by pumpkin, eggplant, lettuce, spinach, leek and green bean juices (from 40.05% to 57.14% of control treatment), with endive and asparagus juices showing the lowest activity. No detectable antimicrobial activity was found when AS-48 was diluted in leek, broccoli, broad bean, mushroom, potato, carrot, tender onion, artichoke, caper, tomato, cucumber and red pepper. However, doubling the bacteriocin concentration revealed some bacteriocin activity (9.16−26.66%) in broccoli, tender onion, broad bean, cucumber and red pepper juices. A pre-heat treatment of 72−98 °C before addition of the bacteriocin resulted in regaining bacteriocin activity in chard, caper, potato, tomato and carrot juices, indicating that the loss in antimicrobial activity of AS-48 was probably due to the activity of proteolytic enzymes that destroyed the bacteriocin. Bacteriocin antimicrobial potential in vegetable juices was also affected by temperature, with AS-48 proving most stable in cabbage juice at 4 °C and 15 °C (Khan, Flint, & Yua, 2010).

In relation with the application of non-thermal technologies to vegetable beverages, Yin, Han, and Liu (2007) proposed the combined use of intense PEF treatments with water-soluble Zn^{2+} concentrations to prevent chlorophyll breakdown without significantly affecting flavour and vegetable quality of spinach purée. They found that increasing field strength, up to 60 kV/cm, increased the green colour of the treated product, which was attributed to the destruction of microorganisms and enzymes that mediate chlorophyll degradative reactions. Field strengths higher than 60 kV/cm appeared to be detrimental to the colour of spinach purée, but whether this negative effect is due to direct action on chlorophylls or to changes in the microstructure of the product, thus affecting chlorophyll protection, has yet to be investigated.

16.10 Conclusion and future trends

Whenever new beverages are developed, it is important to go through every change made in the recipe, packaging and preservation in order to consider the microbial risks. Possible new microbial health risks in beverage production may arise from the increasing importation of ingredients worldwide and from the use of low-acid juices as ingredients. Consequently, deeper studies on process design, optimizing food quality while also satisfying safety constraints, will be a driving force for further evaluation of novel processes and formulations.

Modern beverages typically contain various potential growth enhancers and inhibitors, and their microbial stability is difficult to predict. The future challenge in beverage production is to produce safe and acceptably stable products with minimal

processing, by combining antimicrobial hurdles in order to enhance microbiological quality while maintaining maximum sensory and nutritional quality. Hurdle technology that exploits the synergistic effect of existing natural antimicrobials, together with substances generally regarded as safe and mild physical preservation treatments, is a potential approach for controlling harmful pathogens and spoilage in beverage matrices.

References

Akpinar-Bayizit, A., Ozcan, T., & Yilmaz-Ersan, L. (2012). The therapeutic potential of pomegranate and its products for prevention of cancer. In Alexandros G. Georgakilas, Dr. (Ed.), *Cancer prevention − From mechanisms to translational benefits*. InTech, ISBN 978-953-51-0547-3. Available from http://www.intechopen.com/books/cancer-prevention-from-mechanisms-to-translational-benefits/thetherapeutic-potential-of-pomegranate-and-its-products-for-prevention-of-cance.

Anicuta, S. G., Dobre, L., Stroescu, M., & Jipa, I. (2010). Fourier trasform infrared (FTIR) spectroscopy for characterization of antimicrobial films containing chitosan. *Analele Universitatji din Oradea Fascicula: Ecotoxicologie, Zootehnie si Technologii de Industrie Alimentara*, 1234−1240.

Arroyo, C., Somolinos, M., Cebrián, G., Condón, S., & Pagán, R. (2010). Pulsed electric fields caused sublethal injuries in the outer membrane of *Enterobacter sakazakii* facilitating the antimicrobial activity of citral. *Letters in Applied Microbiology*, *51*, 525−531.

Azizah, A. H., Ruslawati, N. M., & Swee, T. (1999). Extraction and characterization of antioxidant form cocoa by products. *Food Chemistry*, *64*, 199−202.

Barba, F. J., Carbonell-Capella, J. M., Esteve, M. J., & Frigola, A. (2012). Changes in antioxidant capacity of a fruit mixture (orange, mango and papaya) added *Stevia rebaudiana* Bertoni induced by high-pressure processing. In *2nd workshop on food safety: Technology and innovations applied to food safety. Valencia, SPAIN, 5−6 July 2012, book of proceedings* (pp. 235−241), ISBN 978-84-615-9669-0.

Bates, B. (2010). *National diet and nutrition survey: Headline results from year 1 of the rolling programme (2008/2009)*. Food Standards Agency (2010) [Online]. Retrieved on 20-10-2010 from http,://www.food.gov.uk/multimedia/pdfs/publication/ndnsreport0809year1results.pdf.

Belda, C. M., Pina-Pérez, M. C., Jiménez, A., Martinez, A., & Rodrigo, D. (2012). Açai effects on bacterial growth. In *2nd workshop on food safety: Technology and innovations applied to food safety. Valencia, SPAIN, 5−6 July 2012, book of proceedings* (pp. 112−120), ISBN 978-84-615-9669-0.

Bevilacqua, A., Campaniello, D., Speranza, B., Sinigaglia, M., & Corbo, M. R. (2012). Control of *Alicyclobacillus acidoterrestris* in apple juice by citrus extracts and a mild heat-treatment. *Food Control*, *31*(2), 553−559.

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2010). Combining eugenol and cinnamaldehyde to control the growth of *Alicyclobacillus acidoterrestris*. *Food Control*, *21*, 172−177.

Biswas, N., Balac, P., Narlakanti, S. K., Haque, M. D. E., & Hassan, M. D. M. (2012). Identification of phenolic compounds in processed cranberries by HPLC method. *Nutrition and Food Sciences*, *3*(181), 1−6.

Bizri, J. N., & Wahem, I. A. (2006). Citric acid and antimicrobials affect microbiological stability and quality of tomato juice. *Journal of Food Science*, *59*(1), 130−135.

Burdulis, D., Sarkinas, A., Jasutiené, I., Stackeviciené, E., Nikolajevas, L., & Janulis, V. (2009). Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. *Acta Poloniae Pharmaceutica − Drug Research*, *66*, 399−408.

Burt, S. A. (2004). Essential oils: their antibacterial properties and potential applications in foods—A review. *International Journal of Food Microbiology*, *94*, 223−253.

Butkhup, L., Chowtivannakul, S., Gaensakoo, R., Prathepha, P., & Samappito, S. (2010). Study of the phenolic composition of shiraz red grape cultivar (*Vitis vinifera* L.) cultivated in North-Eastern Thailand and its antioxidant and antimicrobial activity. *South African Journal of Enology and Viticulture*, *31*, 89−98.

Ceylan, E., Fung, D. Y. C., & Sabah, J. R. (2004). Antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *E. coli* O157:H7 in apple juice. *Journal of Food Science*, *69*, 102−106.

Coté, J., Caillet, S., Dussault, D., Sylvain, J. F., & Lacroix, M. (2011). Effect of juice processing on cranberry antibacterial properties. *Food Research International*, *44*, 2922−2929.

Criado, M. N., Martínez, A., & Rodrigo, D. (2012). Use of Weibull distribution model to study the inactivation kinetics of peroxidase and polyphenoloxidase from orange, mango and papaya in presence of *Stevia rebaudiana*. In *2nd workshop on food safety: Technologies and innovations applied to food safety* (pp. 317−326), ISBN 978-84-615-9669-0.

Dahan, A., & Amidon, G. L. (2009). Grapefruit juice and its constituents augment colchicine intestinal absorption: potential hazardous interaction and the role of p-glycoprotein. *Pharmaceutical Research*, *26*, 883−892.

Davis, R., & Mauer, L. J. (2010). Fourier transform infrared (FT-IR) spectroscopy: a rapid tool for detection and analysis of foodborne pathogenic bacteria. In A. Menéndez-Vilas (Ed.), *Current research, technology, and education topics in applied microbiology and microbial biotechnology* (pp. 1582−1894).

Del Pozo-Insfran, D., Brenes, C. H., & Talcott, S. T. (2004). Phytochemical composition and pigment stability of açaí (*Euterpe oleracea* Mart.). *Journal of Agricultural and Food Chemistry*, *52*, 1539−1545.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity. *LWT−Food Science and Technology*, *44*(9), 1908−1914.

Élez-Martínez, P., Escolà-Hernández, J., Soliva-Fortuny, R. C., & Martín-Belloso, O. (2004). Inactivation of *Saccharomyces cerevisiae* suspended in orange juice using high-intensity pulsed electric fields. *Journal of Food Protection*, *67*(11), 2596−2602.

Espina, L., Somolinos, M., Ouazzou, A. A., Condón, S., García-Gonzalo, D., & Pagán, R. (2012). Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined treatments of citrus fruit essential oils and heat. *International Journal of Food Microbiology*, *159*(1), 9−16.

Eswaranandam, S., Hettiarachchy, N. S., & Johnson, M. G. (2004). Antimicrobial activity of citric, lactic, malic, or tartaric acids and nisin-incorporated soy protein film against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Gaminara. *Journal of Food Science*, *69*, 79−84.

Fazeli, M. R., Amirmozafari, N., Golbooi nejad, R., & Jamalifar, H. (2007). Antagonistic action of watermelon juice probioticated using different strains of lactobacilli against *Salmonella* Typhimurium. *Iranian Journal of Public Health*, *36*(4), 70−73.

FDA. (2001). *Guidance for Industry: The juice HACCP regulation−questions and answers*. Available at http://www.cfsan.fda.gov/guidance.html.

Ferrante, S., Guerrero, S., & Alzamora, M. S. (2007). Combined use of ultrasound and natural antimicrobials to inactivate *Listeria monocytogenes* in orange juice. *Journal of Food Protection*, *70*, 1850−1856.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2004). Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *Journal of Agricultural and Food Chemistry*, *52*, 6042−6048.

Gaysinsky, S., Davidson, P. M., Bruce, B. D., & Weiss, J. (2005). Growth inhibition of *Escherichia coli* O157: H7 and *Listeria monocytogenes* by carvacrol and eugenol encapsulated in surfactant micelles. *Journal of Food Protection*, *68*(12), 2559−2566.

Hayashi, M., Nasknukool, S., Hayakawa, S., Ogawa, M., Ahmad, A. B., & Nimatulah, A. (2012). Enhancement of antimicrobial activity of lactoperoxidase system by carrot extract and β-carotene. *Food Chemistry*, *130*, 541−546.

Haydock, J., & Hageman, A. (2007). *Açai (*Euterpe oleracea *Mart.), a review of nutrient and polyphenol, content and potential health benefits*. Maastricht, The Netherlands: Department of Health Risk Analysis and Toxicology, Research Institute NUTRIM, University of Maastricht. pp. 1−17. Available at: http://www.soyoufoods.nl/filerep/files/acai%20onderzoek_universieit_Maastricht_1.pdf.

Hodgins, A. M., Mittal, G. S., & Griffiths, M. W. (2002). Pasteurization of fresh orange juice using low-energy pulsed electrical field. *Journal of Food Science*, *67*(6), 2294−2299.

Ingham, S. C., Schoeller, E. L., & Engel, R. A. (2006). Pathogen reduction in unpasteurized apple cider: adding cranberry juice to enhance the lethality of warm hold and freeze-thaw steps. *Journal of Food Protection*, *69*(2), 293−298.

Khan, H., Flint, S., & Yua, P.-L. (2010). Enterocins in food preservation. *International Journal of Food Microbiology*, *141*, 1−10.

Kim, I. S., Yang, M., Lee, O. H., & Kang, S. N. (2011). The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. *LWT-Food Science and Technology*, *44*(5), 1328−1332.

Kumar, K. A., Narayani, M., Subanthini, A., & Jayakumar, M. (2011). Antimicrobial activity and phytochemical analysis of citrus fruit peels−utilization of fruit waste. *International Journal of Engineering Science and Technology (IJEST)*, *3*(6), 5414−5421.

Larrosa, M., Llorach, R., Espin, J. C., & Tomás-Barberán, F. A. (2002). Increase of antioxidant activity of tomato juice upon functionalisation with vegetable by product extracts. *Lebensmittel Wissenschaft und-Technologie*, *35*, 532−542.

Liang, Z., Mittal, G. S., & Griffiths, M. W. (2002). Inactivation of *Salmonella* Typhimurium in orange juice containing antimicrobial agents by pulsed electric field. *Journal of Food Protection*, *65*, 1081−1087.

Lu, X., Rasco, B. A., Jabal, J. M. F., Aston, D. E., Lin, M., & Konkel, M. E. (2011). Investigating antibacterial effects of garlic (*Allium sativum*) concentrate and garlic-derived organosulfur compounds on *Campylobacter jejuni* by using Fourier transform infrared spectroscopy, Raman spectroscopy and electron microscopy. *Applied and Environmental Microbiology*, *77*(15), 5257−5269.

Luckow, T., & Delahunty, C. (2004). Which juice is healthier? A consumer study of probiotic non-dairy juice drinks. *Food Quality and Preference*, *15*(7−8), 751−759.

Mahrour, A., Caillet, S., Nketsia-Tabiri, J., & Lacroix, M. (2003). The antioxidant effect of natural substances on lipids during irradiation of chicken legs. *Journal of the American Oil Chemists' Society*, *80*(7), 679−684.

Martinez-Teruel, A., Sánchez, J., Megías, M. D., & Barbera, J. A. (1998). Using forages and byproducts in dairy cows fams of Murcia region. *Archivos de Zootecnia*, *47*, 33−42.

McNamee, C., Noci, F., Cronin, D. A., Lyng, J. G., Morgan, D. J., & Scannell, A. G. (2010). PEF based hurdle strategy to control *Pichia fermentans, Listeria innocua* and *Escherichia coli* k12 in orange juice. *International Journal of Food Microbiology*, *138*(1−2), 13−18.

Mitic, M. N., Obradovic, M. V., Kostic, D. A., Naskovic, D. C., & Micic, R. J. (2011). Phenolics content and antioxidant capacity of commercial red fruit juices. *Savez Hemijskih Inženjera Srbije*, *65*(5), 611−619.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martin-Belloso, O. (2008a). Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices. *Food Microbiology*, *25*, 479−491.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martín-Belloso, O. (2008b). Inactivation of *Salmonella enterica* ser. Enteritidis in tomato juice by combining of high-intensity pulsed electric fields with natural antimicrobials. *Journal of Food Science*, *23*, M47−M53.

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., et al. (2001). Natural antioxidants from residual sources. *Food Chemistry*, *72*(2), 145−171.

Muanda, F. N., Soulimani, R., Diop, B., & Dicko, A. (2011). Study of chemical composition and biological activities of essential oil and extracts of *Stevia rebaudiana* Bertoni leaves. *LWT-food Science and Technology*, *44*, 1865−1872.

Naidu, A. S. (2000). Lactoperoxidase. In A. S. Naidu (Ed.), *Natural food antimicrobial systems* (pp. 103−132). Boca Raton, FL: CRC Press.

Neida, S., & Elba, S. (2007). Characterization of the acai or manaca (*Euterpe oleracea* Mart.): a fruit of the Amazon. *Archivos Latinoamericanos de Nutricion*, *57*(1), 94−98.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, *156*, 7−17.

Nguyen, P., & Mittal, G. S. (2007). Inactivation of naturally occurring microorganisms in tomato juice using pulsed electric field (PEF) with and without antimicrobials. *Chemical Engineering & Processing*, *46*(4), 360−365.

Oussalah, M., Caillet, S., & Lacroix, M. (2006). Mechanism of action of Spanish Oregano, Chinese cinnamon and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Journal of Food Protection*, *69*(5), 1046−1055.

Parish, M. E. (2007). High pressure inactivation of *Saccharomyces cerevisiae*, endogenous microflora and pectinpethylesterase in orange juice. *Journal of Food Safety*, *18*, 57−65.

Parish, M. E., & Higgins, D. P. (1989). Survival of *Listeria monocytogenes* in low pH model broth systems. *Journal of Food Protection*, *52*, 144−147.

Pina-Pérez, M. C., Martinez, A., & Rodrigo, D. (2012). Cinnamon antimicrobial effect against *Salmonella* Typhimurium cells treated by pulsed electric field (PEF) in pasteurized skim milk beverage. *Food Research International*, *48*, 777−783.

Pina-Pérez, M. C., Silva-Angulo, A. B., Rodrigo, D., & Martinez, A. (2012). A preliminary exposure assessment model for *Bacillus cereus* cells in a milk based beverage: evaluating high pressure processing and antimicrobial interventions. *Food Control*, *26*, 610−613.

Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., & Martín-Belloso, O. (2006). Antimicrobial activity of essential oils on *Salmonella* Enteritidis, *Escherichia coli*, and *Listeria innocua* in fruit Juices. *Journal of Food Protection*, *69*, 1579−1586.

Raybaudi-Massilia, R. M., Mosqueda-melgar, J., & Martin-Belloso, O. (2009). Antimicrobial activity of malic acid against *Listeria monocytogenes, Salmonella* Enteritidis, and *Escherichcia coli* O157:H7 in apple, pear and melon juices. *Food Control*, *20*, 105−112.

Rhodes, P. L., Mitchell, J. W., Wilson, M. W., & Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. *International Journal of Food Microbiology*, *107*, 281–286.

Roussos, P. A. (2011). Phytochemicals and antioxidant capacity of orange (*Citrus sinensis* (L) Osbeck cv. Salustiana) juice produced under organic and integrated farming system in Greece. *Scientia Horticulturae*, *129*, 253–258.

Rupasinghe, H. P. V., & Yu, L. J. (2012). In Yehia El-Samragy, Prof (Ed.), *Emerging preservation methods for fruit juices and beverages, Food additive*. InTech, ISBN 978-953-51-0067-6. Available from http://www.intechopen.com/books/food-additive/emerging-preservation-methods-3-for-fruit-juices-andbeverages.

Ryan, L., & Prescott, S. L. (2010). Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an in vitro digestion. *International Journal of Food Science and Technology*, *45*(6), 1191–1197.

Schauss, A. G. (2010). Açaí (*Euterpe oleracea* Mart.): a macro and nutrient rich palm fruit from the amazon rain forest with demonstrated bioactivities in vitro and in vivo. In *Bioactive food in promoting health: Fruits and vegetables. Chapter 32*. Puyallup, WA: Natural and Medicial Food Division, AIBMR Life Sciences.

Seeram, N. P., Aviram, M., Zhang, Y., Henning, S. M., Feng, L., Dreher, M., et al. (2008). Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. *Journal of Agricultural and Food Chemistry*, *56*(4), 1415–1422.

Su, X., & Souza, D. H. (2011). Grape seed extract for control of human enteric viruses. *Applied and Environmental Microbiology*, *77*(12), 3982–3987.

Touch, V., Hayakawa, S., Yamada, S., & Kaneko, S. (2004). Effects of a lactoperoxidase thiocyanate–hydrogen peroxide system on *Salmonella* Enteritidis in animal or vegetable foods. *International Journal of Food Microbiology*, *93*, 175–183.

USFDA (U.S. Food and Drug Administration). (2006). *Food aditives status list*. Available from URL http//:www.cfsan.fda.gov/~dms/opa-appa.html.

Van Opstal, I., Bagamboula, C. F., Theys, T., Vanmuysen, S. C. M., & Michiels, C. W. (2006). Inactivation of *Escherichia coli* and *Shigella* in acidic fruit and vegetable juices by peroxidase systems. *Journal of Applied Microbiology*, *101*, 242–250.

Vattem, D. A., & Shetty, K. (2002). Solid-state production of phenolic antioxidants from cranberry pomace by *Rhizopus oligosporus*. *Food Biotechnology*, *16*(3), 189–210.

Walker, W., & Phillips, C. A. (2008). The effect of preservatives on *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexanicum* in fruit juice. *Food Control*, *19*, 974–981.

White, B. L., Howard, L. R., & Prior, R. L. (2010). Proximate and polyphenolic characterization of cranberry pomace. *Journal of Agricultural and Food Chemistry*, *58*, 4030–4036.

Wootton-Beard, P. C., & Ryan, L. (2011). Improving public health?: the role of antioxidant-rich fruit and vegetable beverages. *Food Research International*, *44*, 3135–3148.

Wu, Y., Mittal, G. S., & Griffiths, M. W. (2005). Effect of pulsed electric field on the inactivation of microorganisms in grape juices with and without antimicrobials. *Biosystems and Engineering*, *90*, 1–7.

Yin, Y., Han, Y., & Liu, J. (2007). A novel protecting method for visual green color in spinach puree treated by high intensity pulsed electric fields. *Journal of Food Engineering*, *79*, 1256–1260.

Yuste, J., & Fung, D. Y. (2002). Inactivation of *Listeria monocytogenes* Scott A 49594 in apple juice supplemented with cinnamon. *Journal of Food Protection*, *65*(10), 1663–1666.

Using natural antimicrobials to enhance the safety and quality of alcoholic and other beverages

17

M.C. Pina-Pérez, D. Rodrigo, A. Martinez
Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain

17.1 Introduction

Chapter 15 identified the growing interest of consumers in beverages with enhanced sensory and nutritional properties. Bioactive phytochemicals in plants are now recognized for both their antimicrobial and health properties (Negi, 2012). As noted earlier, new 'green additives' are well accepted by concerned consumers who reject the addition of chemicals to food products. Vegetables, fruits, other plant matrices and microbial metabolites are generally recognized as rich sources of bioactive compounds and natural antimicrobials (Kim, Yang, Lee, & Kang, 2011). Natural antimicrobials can inhibit or inactivate spoilage microorganisms responsible for deterioration of food quality (flavour, aroma, colour, texture) and/or can inhibit/delay or inactivate pathogen growth, guaranteeing the food safety of beverages (Pina-Pérez, Martinez, & Rodrigo, 2012; Pina-Pérez, Silva-Angulo, Rodrigo, & Martinez, 2012). At the same time, these natural antimicrobials can positively affect quality characteristics related to food products, including those directly affecting consumer choices (concerning appearance, colour, aroma, taste, freshness and, in general, organoleptically assessed attributes), as well as internal product characteristics not directly detected by consumers (health-promoting compounds, e.g. proteins, phenolic compounds).

The study of the effect of these natural compounds on beverage quality and safety is the main area on which the present chapter focuses, addressing concerns such as (1) how the safety and quality of beverages can be enhanced by means of natural antimicrobials, (2) the effectiveness of these interventions and (3) the factors upon which these interventions depend. This chapter reviews examples of the most important reports that have appeared recently in the scientific literature about the effect of adding antimicrobial compounds obtained from herbs and spices, fruits and vegetables, leaves and bark, animal tissues and microorganisms, which can act alone or by interaction with other non-thermal technologies under the hurdle technology concept.

17.2 Alcoholic beverages

Alcohol has been used throughout history for medicinal purposes; since antiquity, wine has been believed to stimulate appetite and digestion (Williams, 1980). However,

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high alcoholic content, natural vegetable compounds and metabolites resulting from fermentation of these alcoholic beverages are generally sufficient on their own to preserve the microbiological safety of these drinks. Although different physical techniques can be applied to control invasive spoilage microorganisms foreign to the fermentation process selected, addition of natural antimicrobials to these beverages is limited.

17.3 Wine

Among alcoholic beverages, wine is one of the products whose bactericidal activity has been most reported. Among common beverages (cola, beer, milk and wine) acting as antimicrobials per se, wine was the most bactericidal against *Salmonella* Typhimurium, *Shigella sonnei* and *Escherichia coli*. In wine, specifically, resveratrol, a polyphenol produced during grape fermentation, has been reported to be an anti-ageing, anti-carcinogenic and antimicrobial agent (Rotches-Ribalta, Andres-Lacueva, Estruch, Escribano, & Urpi-Sarda, 2012). Under acidic conditions such as those in the human stomach, resveratrol is active and may be linked to inhibition of *Helicobacter pylori*. Many other researchers have confirmed the inactivation of bacteria in wine, and its killing effect is attributed to the ethanol, organic acids, polymeric phenolic fraction and simple phenols that it contains and its low pH (Møretrø & Daeschel, 2004; Rhodes, Mitchell, Wilson, & Melton, 2006; Weisse, Eberly, & Person, 1995).

Enrichment of alcoholic beverages with botanical phytochemicals allows food processors to harness the synergies of their antioxidant functionality and antimicrobial effects. With this objective, Lin, Vattem, Labbe, and Shetty (2005) worked on the enrichment of wine by the addition of 100 μl of cinnamon, green tea, raspberry or peppermint. According to the results obtained, antioxidant and antimicrobial activities were both enhanced by the addition of these phytochemicals, with the antimicrobial effect corresponding to the following order: peppermint > cinnamon > green tea > raspberry. From the point of view of beverage quality improvement, it is worth noting that the soluble phenolic compound content was increased by wine supplementation, from 80 μg/ml (control) to 153 μg/ml (cinnamon), 170 μg/ml (peppermint), 197 μg/ml (green tea) and 224 μg/ml (raspberry). Consequently, specific phenolic profiles in cinnamon, peppermint, green tea and raspberry are suitable as antioxidants and as sources of natural antimicrobial extracts against *H. pylori* when effectively combined with alcoholic beverages.

17.4 Beer

Beer is not a good environment for microbial growth because ethanol and hop compound levels can be high enough to make beer bacteriostatic or bactericidal. Hops are used to impart bitterness and aromatic flavours to beer, and the iso-α-acids present

in hops possess strong antibacterial action against Gram-positive bacteria (Sami et al., 1997; Simpson & Smith, 1992) by microbial *trans*-membrane pH gradient modification with growth inhibition results. In this context, hop-resistant brewing industry isolates are of broader interest, specifically, bacterial isolates belonging to the genus *Pediococcus*, which are well known for their association with contamination of ethanol fermentation processes (e.g. beer). The use of antimicrobial compounds (e.g. hop compounds) by some manufacturers to combat *Pediococcus* contaminants is longstanding, but knowledge about the resistance of pediococci to antimicrobial agents is minimal. Furthermore, lactic acid bacteria as potential spoilage microorganisms in beer at the fermentation stage are of concern to breweries. Natural antimicrobials, alone or in combination with other processes, could be a suitable alternative to improve beer quality, avoiding negative spoiling microorganisms, all the more because conventional time−temperature combinations applied during pasteurization are not effective in eliminating heat-resistant microorganisms without affecting the organoleptic properties of beer or bottle integrity. Galvagno, Gil, Iannone, and Cerrutti (2007) studied the effect of chitosan, hop compounds and nisin against *E. coli*, *Lactobacillus plantarum*, *Pediococcus* spp. and *Bacillus megaterium*. The addition of 0.2 g/ml nisin, 0.1 mg/ml chitosan or 0.3 mg/ml hops selectively inhibited growth of *Pediococcus* spp., producing a reduction over 10,000 times greater than that produced by brewing yeast in a mixed culture. Addition of chitosan and hops to malt barley extract can reduce *E. coli* viability to nearly 1% after incubation for 2 h at 4 °C. Furthermore, addition of chitosan (0.1 mg/ml) to beer leads to no viable cells of the thermoresistant strain *B. megaterium*.

Recently, Franchi, Tribst, and Cristianini (2012) evaluated the antimicrobial capability of nisin, sakacin, lysozyme and hop extracts to reduce beer spoilage, determining the highest effectiveness of nisin (3 mg/l) and lysozyme (100 mg/l) against *Lactobacillus brevis*, *Lactobacillus delbrueckii*, *Acetobacter aceti*, *Listeria innocua*, *Schizosaccharomyces ludwigii and Saccharomyces diastaticus*. Both nisin and lysozyme could be used to control some lactic acid bacteria contaminants in beer, but, according to the authors, other preservation methods should be associated with natural antimicrobials to guarantee the stability of the beer.

17.5 Apple cider

Escherichia coli O157:H7 has also been described as one of the most important microbial contaminants of apple cider. In this context, the addition to apple cider of natural compounds or ingredients as preservatives seems to have good prospects. Essential oils (geraniol, citral, lemon oil, methyl jasmonate, clove, *p*-anisaldehyde and cinnamon) were found to have an antimicrobial effect against *E. coli* O157:H7 in apple cider, at concentration levels in the range 0.01−0.1% (v/v). According to Knight and McKellar (2007), cinnamon oil (minimum inhibitory concentration, MIC 0.025%) and clove oil (MIC 0.075%) showed a marked inhibition of *E. coli* O157:H7 growth with a synergistic effect at acidic pH values (pH 4.5). A possible

explanation is that phenolic compounds present in essential oils (EOs), which act by cell-membrane disruption, are more hydrophobic under acidic conditions and dissolve better in the lipid phase of bacterial membranes. Concentrations of cinnamaldehyde (0%, 0.025%, 0.075% and 0.125% (v/v)) were added to cider (pH 3.60 ± 0.02) to inactivate *E. coli* O157:H7 under refrigerated conditions of 4 °C, 21 days. *Trans*-cinnamaldehyde added to apple cider at concentrations of 0.075 and 0.125% (v/v) completely reduced the *E. coli* O157:H7 load (~5.5 $\log_{10}$ cycles) after 3 days of storage, while 5 days were required to reduce the bacterial load when *trans*-cinnamaldehyde was added at 0.025% (v/v) (Baskaran, malaradjou, Hoagland, & Venkitanarayanan, 2010).

Although *Saccharomyces cerevisiae* and *Saccharomyces uvarum* are the main spoilage cultures added to apple juice and involved in fermentation to produce apple cider, necessary yeast starters to obtain this beverage, the quality of the finished drink could be negatively altered by proliferation of some bacteria and moulds. One of the applications of bacteriocins that has been studied has to do with non-fermented vegetable products, including cider (Settani & Corsetti, 2008). Cider is susceptible to spoilage microorganisms (e.g. *Bacillus licheniformis*) that can seriously affect the quality of the beverage. Accordingly, in order to inactivate undesirable bacteria, Grande et al. (2006) proposed the addition of enterocin AS-48, produced by *Enterococcus faecalis* A-48-32 (formerly *Streptococcus faecalis* subsp. *liquefaciens* S-48). Freshly made apple juice and two commercial apple ciders were tested as substrates to assess the potential of enterocin to inactivate endospore-forming *B. licheniformis*. A 3 μg/ml concentration of enterocin AS-48 was necessary to obtain inhibition in fresh apple juice. According to Grande et al. (2006), bacteriocin AS-48 (*S. faecalis* subsp. *liquefaciens* S-48) increases the heat sensitivity of *B. licheniformis* cells in cider, reducing the time required to achieve complete inactivation of spore-forming bacteria.

Combined treatments have also been applied to apple cider (Ingham & Schoeller, 2002; Yuk & Geveke, 2011). For example, combined mild heat treatment with addition of cinnamon and clove EOs to apple cider significantly reduced the *D*-value and time to 5-log reduction of *E. coli* O157:H7 (Knight & McKellar, 2007).

17.6 Hot drinks

Tea, coffee and cocoa are three of the main hot drinks with inherent antimicrobial and antioxidant properties (Almajano, Carbó, López Jiménez, & Gordon, 2008; Tomás-Barberán et al., 2007). As in the case of dark fruits, the consumption of these beverages on their own or their use as ingredients or supplements in others could have a positive impact on human health. They could also improve beverage functionality, with other added technological functions in newly formulated products (e.g. flavour or antimicrobial applications). These vegetable-derived drinks are valuable sources of catechins and epicatechins with high antioxidant, anti-ageing and antimicrobial characteristics, which make a positive contribution to human health. Matheson, Mainous, Everett, and King (2011) described the benefits derived from consumption of these hot drinks as safe, inexpensive and easily accessible, while they are also an

inexpensive and accessible source of bioactive compounds to be considered by food processors as supplements in the formulation of new beverages.

With regard to tea, green tea, black tea and red tea are some of the best-known teas worldwide. Among the bioactive compounds of teas, catechins, specifically epigallocatechin-gallate, are the most abundant and active antioxidant and antimicrobial compounds in tea (Ellinger, Müller, Stehle, & Ulrich-Merzenich, 2011). In fact, catechins purified from green and black tea have been reported to inhibit the growth of many food-borne pathogens (Friedman, Henika, Levin, & Mandrell, 2006), specifically *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Enteritidis, *E. coli*, *S. sonnei* and *Yersinia* spp. (Perumalla & Hettiarachchy, 2011). Although beverage supplementation with these tea extracts or infusions has scarcely been studied, the potential for their addition as antimicrobials or to enrich new product formulations with antioxidants is clear to the scientific community. Bertipaglia et al. (2008) found that a combined intake of mixed soy:green tea (80:3 g) daily during 45−90 days increased total plasma antioxidant potential. Consequently, it is possible to deduce that mixed beverages that include these two ingredients could have a valuable antioxidant and antimicrobial potential. Perumalla and Hettiarachchy (2011) reported that the compounds rich in polyphenolic and proanthocyanidin that are present in tea extracts have potential antioxidant properties, inhibiting lipid oxidation and warmed-over flavours, and have antimicrobial activity against major food-borne pathogens such as *L. monocytogenes*, *Salmonella* Typhimurium, *E. coli* O157:H7 and *Campylobacter jejuni*. Moreover, with regard to processes commonly applied to preserve beverage quality and safety, it is noteworthy that the antimicrobial activity of green tea remains functional at high temperatures (100 °C/60 min) and even at sterilization temperatures (121 °C/15 min). Among the best-known mixed beverages that include tea, we find lemon green tea and dairy products supplemented with tea. Adding a twist of lemon not only enhances flavour but can also make it easier to absorb antioxidants into the bloodstream. The antimicrobial and antioxidant properties of citrus products, particularly lemon juice, are widely known (O'Bryan, Crandall, & Ricke, 2008), and therefore a combination of these two ingredients could potentially result in additional/synergistic effects regarding microbiological preservation of supplemented beverages. On the other hand, Ferruzzi and Green (2006) studied beverages with milk:tea proportions in the range 10:90 (v/v) to 50:50 (v/v) in terms of catechin content by means of various extraction techniques. These catechin content assessed from a qualitative and quantitative point of view is highly relevant in the aim of tea addition as antimicrobial ingredient in new formulated beverages.

According to Dominguez-Perles, Moreno, Carvajal, and Garcia-Viguera (2011), green tea has also been used as a food matrix to which minimally processed broccoli by-products have been added for the development of a new functional beverage formulated with these two ingredients because they are rich in health-promoting compounds.

Perumalla and Hettiarachchy (2011) go further and point out that synergism in antimicrobial activity of green tea extracts has been observed in combination with organic acids (malic acid, tartaric acid, benzoic acids, etc.), bacteriocins such as nisin or chelating agents such as ethylenediaminetetraacetic acid (EDTA) in various model systems including fresh produce (fruits and vegetables) and raw and ready-to-eat

meat and poultry products. However, the most attractive future for green tea extract involves technologies that actively deliver active components for effective inhibition of pathogens. Research involving nanoscale delivery of the active components present in these natural extracts and using them as a component in a multiple-hurdle approach would enhance beverage safety and quality in addition to providing food processors with alternative 'green' solutions.

Cocoa bean, the seed of *Theobroma cacao*, is known to be rich in polyphenols, such as the flavanol monomers ((+)-catechin and (−)-epicatechin) and procyanidin oligomers (B-type procyanidins that are linked by C4−C8 bonds) (Wollgast & Anklam, 2000). The raw cocoa, after processing, is marketed mainly as chocolate. Various beverages based on cocoa ingredients have been newly formulated, improving their antimicrobial (Pina-Pérez, Rodrigo, & Martínez, 2013), antioxidant (Awe, Fagbemi, Olawunmi, Ifesan, & Badejo, 2013) and functional properties (Puerari, Teixeira- Magalhães, & Schwan, 2012). The antimicrobial properties of polyphenol-rich cocoa (12% minimum content) have already been discussed in Chapter 15, Section 15.3.1 (Pina-Pérez et al., 2013). From a quality point of view, Awe, Fagbemi, Olawunmi, Ifesan, and Badejo (2013) developed a mixed beverage of cocoa, hibiscus flower extract and ginger and evaluated it nutritionally for its antioxidant properties. The results obtained revealed that, although both 100% cocoa and 100% hibiscus flower extract have high antioxidant properties, their combination resulted in increased free radical scavenging potential of the mixed beverage. Cocoa pulp has also been added to kefir beverages, improving fermentation parameters and quality attributes (taste, odour, appearance).

Coffee is one of the most popular beverages in the world and the second largest traded commodity after petroleum. Coffee is cultivated in about 80 countries and generates huge business worldwide. Natural antioxidants, vitamins, enzymes, cellulose, starch, lipids, proteins and pigments are some of the coffee metabolites of high significance to the pharmaceutical and food industries. The most consumed beverage derived from coffee is the flavoured coffee−milk mixture. However, because of the antioxidant and antimicrobial properties of coffee extracts with strong bactericidal action against a broad spectrum of microorganisms (Almeida, Farah, Silva, Nunan, & Glória, 2006), other mixtures are being developed. A vegetable beverage called horchata mixed with iced coffee can be found in the Spanish market, consumed in the summer season. 'Horchata de chufa' is a refreshing, non-alcoholic beverage with a milky appearance, obtained from the tubers of the 'chufa' (*Cyperus esculentus* L.). It is a typical product of Spain and of great economic importance. However, high concern about the natural microflora present in horchata and quality losses in the processed beverage have led to a need to explore new technologies that are less drastic and that can preserve product quality and stabilize the product. The application of pulsed electric field technology (Cortés, Esteve, Frıgola, & Torregrosa, 2005; Selma, Fernández, Valero, & Salmerón, 2003) combined with other hurdles, such as the addition of antimicrobials obtained from natural coffee, offers a promising mixture that should be studied from a microbiological and quality point of view.

17.7 Conclusion and future trends

Whenever new beverages are developed, it is important to go through every change made in the recipe, packaging and preservation in order to consider the microbial risks. Possible new microbial health risks in beverage production may arise from the increasing importation of ingredients worldwide and from the use of low-acid juices as ingredients. Consequently, deeper studies on process design, optimizing food quality while also satisfying safety constraints, will be a driving force for further evaluation of novel processes and formulations.

Modern beverages typically contain various potential growth enhancers and inhibitors, and their microbial stability is difficult to predict. The future challenge in beverage production is to produce safe and acceptably stable products with minimal processing, by combining antimicrobial hurdles in order to enhance microbiological quality while maintaining maximum sensory and nutritional quality. Hurdle technology that exploits the synergistic effect of existing natural antimicrobials, together with substances generally regarded as safe and mild physical preservation treatments, is a potential approach for controlling harmful pathogens and spoilage in beverage matrices.

References

Almajano, M. P., Carbó, R., López Jiménez, J. A., & Gordon, M. H. (2008). Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry*, *108*(1), 55−63.

Almeida, A. A., Farah, A., Silva, D. A., Nunan, E. A., & Glória, M. B. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agriculture and Food Chemistry*, *54*(23), 8738−8743.

Awe, F. B., Fagbemi, T. N., Olawunmi, B., Ifesan, T., & Badejo, A. A. (2013). Antioxidant properties of cold and hot water extracts of cocoa, Hibiscus flower extract. *And Ginger Beverage Blends Food Research International*, *52*(2), 490−495.

Baskaran, S. A., Malaradjou, M. A., Hoagland, T., & Venkitanarayanan, K. (2010). Inactivation of *Escherichia coli* O157:H7 in apple juice and apple cider by trans-cinnamaldehyde. *International Journal of Food Microbiology*, *141*(1−2), 126−129.

Bertipaglia de Santana, M., Mandarino, M. G., Cardoso, J. R., Dichi, I., Dichi, J. B., Camargo, A. E., et al. (2008). Association between soy and green tea (*Camellia sinensis*) diminishes hypercholesterolemia and increases total plasma antioxidant potential in dyslipidemic subjects. *Nutrition*, *24*(6), 562−568.

Cortés, C., Esteve, M. J., Frıgola, A., & Torregrosa, F. (2005). Quality characteristics of horchata (a Spanish vegetable beverage) treated with pulsed electric fields during shelf-life. *Food Chemistry*, *91*(2), 319−325.

Dominguez-Perles, R., Moreno, D. A., Carvajal, M., & Garcia-Viguera, C. (2011). Composition and antioxidant capacity of a novel beverage produced with green tea and minimally-processed byproducts of broccoli. *Innovative Food Science & Emerging Technologies*, *12*(3), 361−368.

Ellinger, S., Müller, N., Stehle, P., & Ulrich-Merzenich, G. (2011). Consumption of green tea or green tea products: Is there an evidence for antioxidant effects from controlled interventional studies? *Phytomedicine*, *18*(11), 903−915.

Ferruzzi, M. G., & Green, R. J. (2006). Analysis of catechins from milk-tea beverages by enzyme assisted extraction followed by high performance liquid chromatography. *Food Chemistry*, *99*(3), 484−491.

Franchi, M. A., Tribst, A. A. L., & Cristianini, M. (2012). Short communication the effect of antimicrobials and bacteriocins on beer spoilage microorganisms. *International Food Research Journal*, *19*(2), 783−786.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2006). Antimicrobial wine formulations against the food borne pathogens *Escherichia coli* O157:H7 and *Salmonella enterica*. *Journal of Food Science*, *71*(7), 245−251.

Galvagno, M. A., Gil, G. R., Iannone, L. J., & Cerrutti, P. (2007). Exploring the use of natural antimicrobial agents and pulsed electric fields to control spoilage bacteria during a beer production process. *Journal Review Argent Microbiology*, *39*(3), 170−176.

Grande, M. J., Lucas, R., Abriouel, H., Valdivia, E., Ben Omar, N., & Maqueda, M. (2006). Inhibition of *Bacillus licheniformis* LMG 19409 from ropy cider by enterocin AS-48. *Journal of Applied Microbiology*, *101*, 422−428.

Ingham, S. C., & Schoeller, N. P. (2002). Acceptability of a multi-step intervention system to improve, apple cider safety. *Food Research International*, *35*, 611−618.

Kim, I. S., Yang, M., Lee, O. H., & Kang, S. N. (2011). The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. *LWT − Food Science and Technology*, *44*(5), 1328−1332.

Knight, K. P., & McKellar, R. C. (2007). Influence of cinnamon and clove essential oils on the *D*- and *z*-values of *Escherichia coli* O157:H7 in apple cider. *Journal of Food Protection*, *70*(9), 2089−2094.

Lin, Y., Vattem, D., Labbe, R., & Shetty, K. (2005). Enhancement of antioxidant activity and inhibition of *Helicobacter pylori* by phenolic phytochemical-enriched alcoholic beverages. *Process Biochemistry*, *40*, 2059−2065.

Matheson, E. M., Mainous, A. G., Everett, C. J., & King, D. E. (2011). Tea and coffee consumption and MRSA nasal carriage. *Annals of Family Medicine*, *9*, 299−304.

Moretro, T., & Daeschel, M. A. (2004). Wine is bactericidal to food-borne pathogens. *Journal of Food Science*, *69*, M250−M257.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, *156*, 7−17.

O'Bryan, C. A., Crandall, P. G., & Ricke, S. C. (2008). Organic poultry pathogen control from farm to fork. *Foodborne Pathogens and Disease*, *5*, 709−720.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts − potential applications in food safety and quality. *Food Research International*, *44*(2011), 827−839.

Pina-Pérez, M. C., Martinez, A., & Rodrigo, D. (2012). Cinnamon antimicrobial effect against *Salmonella* Typhimurium cells treated by pulsed electric field (PEF) in pasteurized skim milk beverage. *Food Research International*, *48*, 777−783.

Pina-Pérez, M. C., Rodrigo, D., & Martínez, A. (2013). Cocoa powder as a natural ingredient revealing an enhancing effect to inactivate *Cronobacter sakazakii* cells treated by pulsed electric field in infant milk formula. *Food Control*, *32*, 87−92.

Pina-Pérez, M. C., Silva-Angulo, A. B., Rodrigo, D., & Martinez, A. (2012). A preliminary expossure assessment model for *Bacillus cereus* cells in a milk based beverage: evaluating high pressure processing and antimicrobial interventions. *Food Control*, *26*, 610−613.

Puerari, C., Teixeira- Magalhães, K., & Schwan, R. F. (2012). New cocoa pulp-based kefir beverages: microbiological, chemical composition and sensory analysis. *Food Research International*, *48*(2), 634−640.

Rhodes, P. L., Mitchell, J. W., Wilson, M. W., & Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. *International Journal of Food Microbiology*, *107*, 281−286.

Rotches-Ribalta, M., Andres-Lacueva, C., Estruch, R., Escribano, E., & Urpi-Sarda, M. (2012). Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets. *Pharmacology Research*, *66*, 375−382.

Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., et al. (1997). Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. *Journal of Fermentative Bioengineering*, *84*, 1−6.

Selma, M. V., Fernández, P. S., Valero, M., & Salmerón, M. C. (2003). Control of *Enterobacter aerogenes* by high-intensity, pulsed electric fields in horchata, a Spanish low-acid vegetable beverage. *Food Microbiology*, *20*(1), 105−110.

Settani, L., & Corsetti, A. (2008). Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology*, *121*, 123−138.

Simpson, W. J., & Smith, A. R. (1992). Factors affecting antibacterial activity of hop compounds and their derivatives. *Journal of Applied Bacteriology*, *72*, 327−334.

Tomás-Barberán, F. A., Cienfuegos-Jovellanos, E., Marin, A., Muguerza, B., Gil-Izquierdo, A., Cerda, B., et al. (2007). A new process to develop a cocoa poder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of Agricultural and Food Chemistry*, *55*, 3926−3935.

Weisse, M. E., Eberly, B., & Person, D. A. (1995). Wine as a digestive aid: comparative antimicrobial effects of bismuth salicylate and red and white wine. *British Medical Journal*, *311*(7021), 1657−1660.

Williams, S. (1980). The use of beverage alcohol as medicine, 1790−1860. *Journal of Studies on Alcohol*, *41*, 543−566.

Wollgast, J., & Anklam, E. (2000). Polyphenols in chocolate: is there a contribution to human health? *Food Research International*, *33*, 449−459.

Yuk, H. G., & Geveke, D. J. (May 2011). Nonthermal inactivation and sublethal injury of *Lactobacillus plantarum* in apple cider by a pilot plant scale continuous supercritical carbon dioxide system. *Food Microbiology*, *28*(3), 377−383.

Using natural antimicrobials to enhance the safety and quality of poultry

18

M. Singh,[1] *J. Smith,*[2] *M. Bailey*[1]
[1] Purdue University, West Lafayette, IN, USA; [2] Auburn University, Auburn, AL, USA

18.1 Introduction

The growth in global poultry production increases the need for consistently safe, wholesome, and nutritious products. This issue is further propagated by continuously evolving consumer requirements for poultry that is produced using natural products, particularly those which can be applied throughout the poultry production continuum all the way from the farm, to transportation, and continuing into the processing plants. Furthermore, items with organic labels are becoming ever more popular with consumers due to their natural and preservative-free characteristics (Sirsat, Muthaiyan, & Ricke, 2009). Generally, the "natural" labeling term has been used on products that have no artificial ingredients or added colors and are minimally processed. According to the United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS), such foodstuffs must be accompanied with a statement explaining the usage of the term (e.g., no artificial ingredients; minimally processed) (USDA-FSIS, 2006, 2013). Despite efforts from the industry and the USDA-FSIS, poultry and its products appear twice in the top 10 list of risk foods for human food-borne illnesses, once paired with campylobacteriosis and then associated with salmonellosis (Batz, Hoffman, & Glenn-Morris, 2012). The poultry industry is a vertically integrated enterprise, where total control starts from the breeder stock and continues through the hatcheries, farm practices, transportation, processing plants, and finally onto further processing plants. Growing food safety concerns and the impact of using antimicrobial on the quality of poultry meat products has generated the need for development and implementation of natural antimicrobials. This chapter discusses the need for natural antimicrobials, their impact on safety and quality of poultry products, and their use in the poultry industry.

18.2 Food safety and its role in food quality

Food products of animal origin are increasingly scrutinized for their contribution to human illnesses, with poultry products being no exception. The Centers for Disease Control and Prevention (CDC) has reported that more deaths have been attributed to poultry products in recent years than to any other food commodity (Painter et al.,

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2013). The nutrient-rich environment of poultry carcasses and parts lends itself to rapid microbial growth; moreover, poultry skin is a complicated and extremely intricate surface, making it very difficult to completely eliminate all microbiological contamination on such surfaces. The safety of poultry products cannot be compromised for the sake of their quality, and measuring the impact of applying antimicrobials on product quality is often overlooked. The impact on organoleptic properties of products is of prime importance to processors because it has a significant impact on consumer preference. Given that fresh poultry products are highly perishable, shelf-life investigations can yield a tremendous amount of knowledge regarding the appropriate application of antimicrobials on poultry products.

18.2.1 Poultry processing

Commercial poultry husbandry has been extensively studied to optimize growth rates while maintaining the highest quality and welfare conditions possible. Poultry processing specifically involves converting the muscle tissues of live broiler chickens to meat intended for human consumption. This is a highly automated and multistep process that originates with commercially housed broilers and culminates with many different products in numerous market-ready forms. The processing plant is the point at which interventions, mainly antimicrobials, may be applied in order to reduce the natural microflora on fresh poultry carcasses.

Poultry processing begins in the grow-out houses. Feed and water are withdrawn here so the intestinal tract is devoid of any undigested material at the time of processing. Ideally, this time is limited to between 8 and 10 h prior to primary processing. At the broiler processing plant, the primary processing begins with unloading the carcasses from the transportation vehicle. They are then hung onto individual shackles in an environment with dark lighting to minimize bird activity, followed by stunning using a saline solution to render them insensitive to pain, killed by bleeding followed by scalding and defeathering. Usually, mechanical removal of head, feet, and viscera follows and carcass chilling ensues. An inside-outside bird washer (IOBW) is commonly used prior to the chilling process. Antimicrobials can be used in the IOBW, but it is important to note that the mechanical force from the sprayers can also account for bacterial reduction. Chillers and IOBWs have been studied extensively in laboratory and commercial settings and are typically steps in which processors are able to achieve bacterial reduction at higher rates compared to other steps when these processes are managed appropriately.

The most effective method to achieve reductions in poultry-borne bacterial pathogens during commercial poultry procurement is a multi-hurdle approach, where many intervention points are used along the processing line to reduce the chances of pathogens surviving. This approach is unique in each poultry processing plant and can utilize multiple IOBWs and/or dip tanks before and after chilling. Carcass chilling also represents an opportunity to reduce and/or eliminate poultry-borne pathogens, utilizing a countercurrent system whereby water flows in the opposite direction to carcass movement and therefore continuously exposes carcasses to cleaner water.

18.2.2 General recognized as safe (GRAS) compounds

The Food and Drug Administration (FDA) recognizes substances intentionally added to food as food additives, and also further classifies some of these substances as Generally Recognized As Safe (GRAS). Obtaining GRAS status alleviates the need for a substance to be classified as a food additive under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act, thus further eliminating the need to be subjected to premarket review and approval by the FDA.

Criteria for achieving GRAS status comes through the "same quality and quantity of scientific evidence" required for non-GRAS substances (FDA, 2013). This comes from published scientific work, unpublished studies, and further data and information regarding the substance itself. Furthermore, GRAS status can be obtained through experience based on common use in foods accompanied by "substantial history of consumption for food use by a significant number of consumers" (FDA, 2013). Additionally, the FDA has defined "safe" to mean "that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use" (21 CFR 170.3(i)). A substance may also be considered GRAS for one use and not for another use, depending on the product's application. Irrespective of the GRAS status of an antimicrobial used in poultry production, the primary concern for regulatory agencies is the intended use of the product.

18.3 Pre-harvest use of natural antimicrobials

Antibiotics have been used extensively over the past half-century as growth-promoting feed additives as well as control measures for pathogens. The demand for antibiotic-free birds, both from regulatory pressure as well as from consumers, has had a massive influence on the poultry industry, especially in the European Union, where non-therapeutic use of antibiotics has been banned since 2006 (Brenes & Roura, 2010; Tellez, Pixley, Wolfenden, Layton, & Hargis, 2012). There are two main reasons for the use of natural antimicrobial replacements for these non-therapeutic antibiotics. The first reason is from a perspective of food safety, namely, that control of food pathogens will be most effective if efforts to prevent product contamination are applied throughout the food production process, including during pre-harvest (Sofos, 2008). Therefore, with the reduced use of antibiotics as control measures comes the need for alternative strategies to control pathogens during grow-out. The second reason is the need to maintain bird health and feed efficiency by finding substitutes that can provide protection for the birds from pathogens like *Clostridium perfringens* and *Escherichia coli*, and substitutes that can also induce a similar growth-promoting effect. Additionally, though regulations in the United States prevent the use of non-therapeutic doses of antibiotics in birds raised to be marketed as organic, it is required that flocks be treated with antibiotics in the event of a disease outbreak that cannot be treated using alternative methods. In this case, treated birds can no longer be marketed as organic (Griggs & Jacob, 2005). To prevent this reduction in product quality, adequate alternative

treatments must be explored. Pre-harvest controls that are currently being utilized and researched include probiotics, prebiotics, organic acids, and plant extracts.

18.3.1 Probiotics

Probiotics have been defined as "live microorganisms of non-pathogenic and non-toxic nature, which when administered through the digestive route, are favorable to the host's health" (Kabir, 2009). These live microorganisms establish themselves within the digestive tract of the host animal and provide numerous benefits including protection from pathogens by competitive exclusion and maintaining proper balance of intestinal microflora, inducing beneficial changes to metabolism, improving efficiency of feed use, and inducing immune response (Gaggía, Mattarelli, & Biavati, 2010; Kabir, 2009; Revolledo, Ferreira, & Mead, 2006). Typically administered as feed ingredients or as additives to drinking water, probiotics have the ability to provide some of the growth-promoting effects seen with antibiotic use. Species commonly used for this purpose are natural to the digestive tract (mostly lactic acid bacteria) and are non-pathogenic to healthy animals (Tellez et al., 2012). These include numerous *Lactobacillus* and *Bifidobacterium* species, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enteroccocus faecalis*, *E. coli*, and some *Bacillus* species (Kabir, 2009; Tellez et al., 2012). In addition, some species of fungi, including *Saccharomyces cerevisiae* and *Aspergillus oryazae*, have been used for this purpose as well, with limited success (Kabir, 2009). Schneitz, Hakkinen, Nuotio, Nurmi, and Mead (1990) developed the first successful commercial probiotic (under the tradename Broilact), which was a mixture of 22 anaerobic bacilli and 10 facultative anaerobes isolated from healthy adult hens. Broilact was shown to prevent colonization and infection of chicks by *S*. Enteritidis, *S*. Typhimurium, *Campylobacter jejuni*, and *E. coli* O157:H7 (Edens, 2003). Since this experiment, there have been numerous studies involving the control of *Salmonella* colonization in poultry using lactic acid bacteria-based probiotics (Higgins et al., 2007; Vicente et al., 2008; Wolfenden et al., 2007). Use of *Bacillus subtilis* strain PY79 was able to reduce shedding of *S*. Enteritidis in experimentally infected birds (although colonization of internal organs was not significantly different from the controls) and was able to reduce colonization and shedding of *C. perfringens* over the course of the trial (La Ragione & Woodward, 2003). Few studies have investigated the use of probiotics against *C. jejuni* colonization and shedding. Chaveerach, Lipman, and van Knapen (2004) showed *in vitro* bactericidal effects of *Lactobacillus* strain P93 against 10 isolates of *Campylobacter*, and Morishita, Aye, Harr, Cobb, and Clifford (1997) used a commercial mixture of *Lactobacillus* species to achieve a 70% reduction of *C. jejuni* in chicks. Santini et al. (2010) demonstrated significant activity of *Bifidobacterium. longum* strain PCB 133 against *Campylobacter* when administered to chicks orally for 15 days.

18.3.2 Prebiotics

Prebiotics are feed additives that are not readily digested by the bird, such as complex carbohydrates, certain peptides, proteins, lipids, and lactose (Hajati & Rezaei, 2010).

Of these, the most frequently studied compounds are the fructo-oligosaccharides (FOS) found in plants such as onions, artichokes, bamboo shoots, bananas, chicory, and some cereal grains (Griggs & Jacob, 2005; Hajati & Rezaei, 2010). As they are non-digestible, these prebiotics provide substrates for commensal bacteria in the gastrointestinal tract and in consequence help to balance microfloral populations in favor of beneficial organisms and to the exclusion of pathogens (Callaway et al., 2008; Hajati & Rezaei, 2010). Additionally, Seifert and Watzl (2007) demonstrated immuno-modulatory effects of dietary FOS, which could benefit intestinal health by alleviating inflammation. Using prebiotics in combination with probiotics is called "synbiotics." This combination can work synergistically to exclude pathogens from the gastrointestinal tract and reduce disease (Callaway et al., 2008; Collins & Gibson, 1999). Bailey, Blankenship, and Cox (1991) reported that diets containing 0.75% FOS in addition to probiotic flora were able to reduce *Salmonella* colonization rate to 12% fewer birds than the control. Xu, Hu, Xia, Zhan, and Wang (2003) demonstrated that 0.4% dietary FOS achieved a reduction in *E. coli* in the rectum and small intestine in favor of *Bifidobacterium* and *Lactobacillus*. However, other studies have had inconsistent results (Griggs & Jacob, 2005). In addition, although there appear to be several benefits to the use of prebiotics in poultry, these compounds are currently expensive to use on a commercial scale (Callaway et al., 2008).

18.3.3 Organic acids

Control of pathogenic or non-beneficial bacteria during pre-harvest has been attempted using organic acids added to the feed or to drinking water; these experiments have had mixed results (Griggs & Jacob, 2005). Gunal, Yayli, Kaya, Karahan, and Sulak (2006) found that a mixture of organic acids including propionic and formic acid, when added to feed, reduced total bacterial counts in the ileum and ceca, specifically Gram-negative organisms. This may or may not be positive, as some Gram-negative bacteria, such as *E. coli*, can provide probiotic activity (Behnsen, Deriu, Sassone-Corsi, & Raffatellu, 2013). In another study, adding formic and propionic acid to the diet lowered the lactic acid content in the crop (Thompson & Hinton, 1997). These researchers concluded that the added acid may have killed natural lactic acid bacteria. Hinton and Linton (1988) reported that formic acid-treated feed decreased *Salmonella* colonization of birds as long as treatment began with newly hatched chicks. If treatments were started at a later time, this reduction was not observed. In the same experiment, a propionic acid added to feed was able to reduce infection in chickens when feed was inoculated with low levels of *S*. Kedougou. Al-Chalaby, Hinton, and Linton (1985) found that a commercial propionic acid mixture could eliminate *Salmonella* in drinking water; however, it did not reduce *Salmonella* levels in litter, cloacal swabs, or cecal samples. Certain ratios of formic, acetic, and proprionic acids have been shown to lower the spread of *Campylobacter* throughout a flock when added to the drinking water (Chaveerach et al., 2004). The use of caprylic acid as a feed additive was able to lower *C. jejuni* colonization of young birds and reduce *C. jejuni* in previously colonized 42-day-old birds (de los Santos et al., 2008, 2009), although an inconsistent effect was observed when caprylic acid was added to drinking water (Metcalf et al., 2011).

18.3.4 Plant extracts

Various botanical extracts such as essential oils have been tested as feed additives for poultry and other livestock. The majority of *in vivo* studies utilizing essential oils have focused mainly on the growth-promoting characteristics of these extracts. There is little *in vivo* evidence explaining the mechanism of action for growth-promoting effects of essential oils, but it is thought to occur primarily through modulation of gastrointestinal microflora and inhibiting colonization by pathogens, improvement in digestion through increased enzyme activity, prevention of tissue oxidation, and immunostimulation resulting in reduced inflammation (Brenes & Roura, 2010; Windisch, Schedle, Plitzner, & Kroismayr, 2008). From a food safety standpoint, the main interest in using essential oils as feed additives is to reduce the prevalence of pathogens during pre-harvest. The antimicrobial properties of various essential oils and their components are well known and much research exists demonstrating these properties *in vitro* (Griggs & Jacob, 2005). Antimicrobial activity against *E. coli* has been shown in essential oils from thyme and cloves (Farag, Daw, Hewedi, & El-Baroty, 1989); oregano and cinnamon (Friedman, Henika, & Mandrell, 2002); and garlic (Ross, O'Gara, Hill, Sleightholme, & Maslin, 2001). Activity against *S*. Typhimurium has been shown in thymol and eugenol, which are components of thyme and clove essential oils, respectively (Karapinar & Aktuğ, 1987). Carvacol, a component of oregano essential oil, has also been shown to have activity against *S*. Typhimurium (Helander et al., 1998), as has garlic oil (Ross et al., 2001). Friedman et al. (2002) reported the *in vitro* antimicrobial properties of 96 essential oils and 23 components against food-borne pathogens, including *C. jejuni*, *E. coli* O157:H7, *Listeria monocytogenes*, and three serovars of *Salmonella enterica*. Despite the exhaustive literature characterizing inhibitory abilities of plant extracts, the need for *in vivo* experimentation remains and must be addressed before the usefulness of these compounds in a commercial setting can be determined.

Despite efforts to control pathogens during pre-harvest, there is considerable research to be done involving this subject. Ultimately, it remains very difficult to control pathogens during the live stages of production primarily because a total comprehension of the complex relationships involved in the spread and persistence of pathogens remains elusive. Some confounding factors include incomplete understanding of pathogen reservoirs and sources, inability to effectively identify asymptomatic carriers of pathogens like *E. coli* O157:H7, the ubiquitous nature of some pathogens, pathogens present at low levels or low prevalence and sporadic shedding of these pathogens, large amounts of interfering background flora, ineffective methods for field testing, and the cost-prohibitive nature of existing control interventions (Sofos, 2008). These factors will need to be addressed in order to increase the effectiveness of pre-harvest intervention.

18.4 Antimicrobials for use on poultry products

The selection of an antimicrobial for use in food production begins when the overall safety and quality of the finished product is considered. Additionally the regulatory

status of the product must be addressed and its intended use defined. Next, the product needs to be evaluated with respect to all potential microbiological hazards for a product. Each of these elements must be considered before a product can be applied for use in food production, especially poultry processing. Furthermore, the research and investigation of intervention strategies to more efficiently produce safe and wholesome meat and poultry products with minimal water use is especially necessary to facilitate the economic production of poultry products.

In 1996, the USDA-FSIS implemented a "zero tolerance" pre-chill performance standard for visible fecal material as part of their final ruling on the implementation of the Hazard Analysis and Critical Control Point (HACCP) systems for meat and poultry production. HACCP is a systematic and preventative approach to food safety that identifies physical, chemical, and microbiological hazards. HACCP utilizes Critical Control Points (CCPs) where hazards can either be reduced or eliminated to monitor process control (USDA, 1996).

Poultry processors are faced with many challenges within the plant, particularly those imposed in the form of USDA-FSIS Performance Standards. From July 2007 to June 2008, the USDA-FSIS conducted a Nationwide Microbiological Baseline Data Collection Program, examining 6550 carcass rinse samples, which revealed that the post-chill prevalence of *Campylobacter* was 40.23% positive. This data also showed that 99.8% of the samples taken were below 3 $\log_{10}$ CFU/ml. This sampling led to the implementation of *Campylobacter* performance standards on July 1, 2011 (75 FR 27,288). For young chickens, post-chill *Campylobacter* should not be greater than 10.4% positive. The sampling technique used utilizes a 1 ml portion of a 400 ml carcass rinse microbiologically plated onto four separate agar plates (0.250 ml per plate) to detect higher levels of contamination. A carcass is considered positive for *Campylobacter* if at least one colony appears on one of the four Campy Cefex plates.

Along with the new *Campylobacter* performance standards, the USDA-FSIS also issued updated *Salmonella* performance standards (USDA, 2009). While these performance standards seem to be logical means to reduce cases of human salmonellosis, and the new *Campylobacter* performance standards by the same logic should reduce human campylobacteriosis, these rates have seen very little impact over the course of the introduction of these performance standards (Russell, 2012). Antimicrobials that consistently control both *Campylobacter* and *Salmonella* spp. have the potential to affect the overall breadth of poultry processing.

18.4.1 Chemical antimicrobials

Chemical antimicrobial treatments are evaluated for many different factors and can be used in a variety of applications on poultry products. Additionally, worker safety and production of harmful effluents should be monitored and minimized for environmental concerns; another very important concern is that a chemical antimicrobial should not have any organoleptic effects on the products they are being used to treat.

18.4.2 Water

The most essential element of human life, which covers 70% of the earth's surface, water is also important in poultry processing. It is the primary vehicle used for eliminating unwanted feathers, fecal matter, and microorganisms within poultry plants (Burton, Cumby, & Tinker, 2004). After the implementation of HACCP in 1996, poultry processors were troubled by establishing measures to comply with the pre-chill zero visible fecal contamination regulations, which resulted in increasing carcass wash systems and some plants even increased water use by as much as 50% (Jackson, Curtis, Carawan, Keener, & Taylor, 1999). The primary source of concern to processors stemmed from the idea that the application of pre-chill performance standards ignored the antimicrobial effects of the immersion chilling process (Bilgili, Waldroup, Zelenka, & Marion, 2002).

Industry surveys have revealed that the average water use amounts to approximately 26 l/bird, and it is not uncommon for plants to process over 1 million birds each week (Northcutt & Jones, 2004). In the United States, the primary site for water use is during immersion chilling, which can have capacities of up to 40,000 gallons. The primary goal of chilling is to reduce the internal temperature of the carcass to below 4.4 °C within 4 h of slaughter (USDA, 2008); however, this is also a time when antimicrobials are frequently added to this water to further reduce the pathogen load of carcasses. Using water alone during chilling will achieve an overall washing effect on carcasses due to the extended dwell time of up to 2 h and agitation while in the immersion chiller. However, immersion chilling is an especially important site of cross-contamination because of the volume of carcasses that come into contact with one another and the extended duration of this process. Many immersion chillers are designed to apply a countercurrent flow to minimize the extent of cross-contamination. This system operates by pumping clean water into the chiller close to the exit so that the current moves opposite to the processing line. Therefore, carcasses are exposed to gradually cleaner water as they move through the chiller, increasing the performance of the washing effect. Because of its greater washing potential, the countercurrent design is considered more effective at reducing total bacteria load on carcasses (Conner, Davis, & Zhang, 2001). Additionally, Bashor et al. (2004) suggested that using multiple IOBW sequentially is more effective than just one IOBW. A single IOBW did not significantly reduce aerobic bacteria but inoculated *Salmonella* Typhimurium were significantly reduced (Yang, Li, & Slavik, 1998).

18.4.3 Hot water and steam

Hot water is currently part of the multistage scalding process, which prepares the outer surface of the carcass for removal of the feather follicles. This process also assists in overall removal of extraneous material from the carcass surface but has also been investigated for reduction of bacterial loads on carcasses. Using hot water and steam pre-chill is the most logical application of these techniques, since carcasses are required to be below 4.4 °C post-chill to meet USDA-FSIS performance standards (USDA, 2008).

Patrick, Goodwin, Collins, Wyche, and Love (1972) compared steam to hot water scalding, and while the steam technique yielded lower total plate counts than hot water immediately after scalding, there was no difference observed in the two methods after chilling. Exposure to pasteurizing steam (90 °C) has shown the ability to significantly reduce total viable counts of *Enterobacteriaceae* and *Campylobacter* by approximately 1 $\log_{10}$ CFU/g on whole broiler carcasses after 24 s of exposure; however, this exposure did damage the outer layer of the skin (Whyte, McGill, & Collins, 2003). Furthermore, Corry et al. (2007) showed reductions of 1.66 $\log_{10}$ CFU/cm^2 *C. jejuni* AR6 on inoculated broiler carcasses using only 30 s exposure at 75 °C, with no negative effects reported on carcass appearance.

18.4.4 Organic acids

The use of organic acids on food products is based upon the concept of creating an acidic environment less favorable to bacterial survival. These acids are known as weak acids, referring to their pH-dependent dissociation in water. A weak acid tends to dissociate in water until the pH falls below the acid's pK_a, at which point it will protonate, lowering its polarity and therefore increasing its ability to diffuse across the cell membrane. In consequence, reduction in pH of the food matrix or surrounding medium will enhance the concentration of protonated acid, raise the concentration of acid in the cytoplasm, and thus increase antimicrobial activity (Mani-López, García, & López-Malo, 2012). Such acids have been used in surface applications and can also be incorporated into products themselves. For whole carcasses both spray and dip applications have been explored, and for cut-up portions many other uses have been documented. The efficacy of organic acids against target microorganisms varies with respect to concentration, contact time, temperature, and the design of experimental parameters (Dickson & Anderson, 1992).

An important factor to consider is the protective effect observed against cells attached to or embedded in carcass skin. Attached cells are more resistant to the bactericidal effects of organic acids, so higher concentrations are required to kill them. In the case of *S*. Typhimurium, concentrations as high as 4% may be necessary to cause adequate antimicrobial effect against attached cells (Tamblyn & Conner, 1997a). Using such high concentrations of organic acids is associated with certain disadvantages. Undesired organoleptic changes in color or flavor may be induced, and the expense of using high acid concentrations may be cost-prohibitive (Tamblyn & Conner, 1997a). The additional use of some transdermal synergists has been shown to increase the effectiveness of organic acids in certain applications. Tamblyn and Conner (1997a) found that both SPAN-20 and SDS, when added to 0.5% concentrations of various organic acids, significantly increased antimicrobial activity against attached *S*. Typhimurium. Transdermal synergists, which are typically surfactants, are thought to increase the activity of organic acids because of their emulsifying properties. By inducing the mixture of water and lipid phases, these compounds can augment transmission of organic acids to bacterial cells attached to the carcass, increasing the acid's effectiveness so that lower concentrations can be used (Tamblyn & Conner, 1997a).

18.4.4.1 Acetic acid

Also known as food-grade vinegar, metabolic byproducts such as GRAS-status acetic acid (CH_3COOH) are approved for use at multiple stages in poultry processing (USDA, 2012). Experimental applications of acetic acid (0.60%) as a pre-chill dip (10 min) reduced Enterobacteriaceae counts while not impacting total aerobic bacteria, but this produced a yellowing of skin with protruding feather follicles (Dickens & Whittemore, 1994). Conversely, Dickens and Whittemore (1997) did not observe changes in carcass appearance when acetic acid (1%) was applied during picking (30 s) but were able to observe significant reductions in total aerobic bacteria after scalding. Tamblyn and Conner (1997b) investigated the bactericidal activity of acetic acid (among other organic acids) against *S*. Typhimurium attached to broiler chicken skin during scalding, simulated chill, and post-chill dip. This study demonstrated a linear association between increasing acid concentration (0.5, 1, 2, 4, and 6%) and log CFU reduction. However, using this skin-attachment model, it was apparent that greater concentrations of acetic acid were required to achieve similar log CFU reductions of attached cells when compared to suspended cells, indicating greater resistance to organic acids in attached cells. Bilgili, Conner, Pinion, and Tamblyn (1998) were able to show that many organic acids, including acetic acid, are able to reduce lightness (L*) and increase yellowness (b*) values. A novel approach combining Modified Atmosphere Packaging (MAP; 70% CO_2/30% N_2) and acetic acid decontamination suppressed pseudomonad growth and extended the lag phase of both lactobacilli and enterobacteria after 7 days of storage (Jimenez, Salsi, Tiburzi, Rafaghelli, & Pirovani, 1999).

18.4.4.2 Lactic acid

Lactic acid is a hygroscopic, syrupy liquid frequently used in the production of jams, jellies, and other confectionary products, and it also has the ability to prevent discoloration of fruit such as apples (Doores, 2005). The efficacy of lactic acid against Gram-negative bacteria such as *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *S*. Typhimurium is based on the ability to permeate the outer membrane and may act as a potentiator of other antimicrobial substances (Alakomi et al., 2000). Such acids are used, having GRAS status approval, successfully in poultry processing as a dip and in chiller and scalder water.

As both a spray and dip application, a 1% lactic acid treatment was able to reduce aerobic bacteria, *Pseudomonas*, and *Lactobacillus* counts to near undetectable levels with high sensory acceptability immediately after treatment. The frequency of *Salmonella* recovery was also reduced at a greater rate using the aforementioned dip method to the same degree as trisodium phosphate (Okolocha & Ellerbroek, 2005). Levels of inoculated *S*. Typhimurium were also significantly reduced using lactic acid (1%) in chill water and as a post-chill dip and as a pre-chill dip (2%); however, mild skin discoloration was reported for all carcasses subjected to a lactic acid treatment during picking (Izat, Colberg, Thomas, Adams, & Driggers, 1990). Hwang and Beuchat (1995) showed that a wash solution of 1% lactic acid reduced *Salmonella* spp., *L. monocytogenes*, and psychrotrophic counts more effectively than a water

treatment alone. After treatment with lactic acid (1% or 3%) and storage under refrigerated (4 °C for 10 d) and frozen (−18 °C for 6 months) conditions, survival of inoculated *S*. Enteritidis was significantly lower than carcasses treated with only a water wash (Cosansu & Ayhan, 2012). The effectiveness of lactic acid can also benefit from synergistic compounds. The addition of propylene glycol to lactic acid was reported to completely remove *Salmonella* from chicken carcasses (Izat et al., 1990).

18.4.4.3 Citric acid

Citric acid has numerous applications throughout the food industry. With its mildly sour and pleasant taste, it is frequently used to enhance the flavor of citrus-based foods (Doores, 2005) and is also used for pH control when canning certain low-acid foods such as tomatoes. For poultry processing, citric acid is mainly used along with hydrochloric acid to control the pH of processing water but does not have to be labeled as such (USDA, 2012). Another novel application of citric acid approved by the USDA-FSIS is on the soaker pads used in retail packaged cuts of fresh poultry in an attempt to reduce the microbial load of the purge that accumulates on such pads (USDA, 2012). The incorporation of citric acid at both 3% and 6% into the diets of broilers decreased feed intake, body weight gain, and carcass yield, and also improved feed conversion ratio; however, no additional benefit was observed with the 6% level when compared to a normal basal diet (Nourmohammadi, Hosseini, & Farhangfar, 2010). Incorporation of citric acid helped decrease the undesirable, inherent pink color of ground turkey deli loaves but increased or showed no effect on the pinkness of intact turkey breasts (Sammel & Claus, 2003). Similar to other organic acids, use of citric acid to reduce pathogen load may also benefit from the addition of transdermal synergists. Restaino, Frampton, Bluestain, Hemphill, and Regutti (1994) demonstrated more than a 5-log reduction in suspended *S*. Typhimurium cells using a combination of citric acid, ethylenediaminetetraacetic acid (EDTA), and sodium dodecyl sulfate (SDS). However, it is unclear whether or not this effect would carry over to carcasses or other poultry products.

18.4.4.4 Other organic acids

There are several other acids that are approved as GRAS substances for use in food products. These include propionic, succinic, malic, and tartaric acids. Few studies have characterized the ability of propionic and succinic acids to reduce food-borne pathogens on poultry and meat products during processing (Mani-López et al., 2012). Milillo and Ricke (2011) showed that sodium propionate, a salt of propionic acid, achieved a better minimum inhibitory concentration against *S*. Typhimurium in model raw chicken medium than salts of citric and lactic acids. Succinic acid (sprayed at 1% concentration) has been used to reduce *S*. Typhimurium by 60% on experimentally inoculated carcasses (Thomson, Banwart, Sanders, & Mercuri, 1967), although Cox, Mercuri, Juven, Thomson, and Chew (1974) found that higher concentrations of as little as 3% negatively impacted organoleptic properties. The activities of malic and tartaric acids against *S*. Typhimurium on chicken breast skin were investigated by Tamblyn and Conner (1997b). In this study, as little as 0.5% malic acid applied under simulated chill and scald conditions was able to reduce *S*. Typhimurium

by over 1 log CFU. Similar results were obtained for tartaric acid after simulated scald. Other types of organic acids that can be used to reduce pathogens during processing include alkaline salts of fatty acids, such as lauric acid. Hinton, Cason, Buhr, and Liljebjelke (2009) reported a significant reduction in total bacterial counts from poultry carcass rinses after a 5 s wash with a mixture of 2% lauric acid and 1% potassium hydroxide. After 15 s, a significant reduction in *S*. Typhimurium was observed, and no *E. coli* was recovered after at least a 15 s wash. Levulinic acid (3%) has also been used (in combination with 2% SDS) to reduce *Salmonella* on chicken wings and in water contaminated with chicken feces (Zhao, Zhao, & Doyle, 2009).

Organic acids have much potential as inhibitors of pathogens during poultry processing. A major advantage of organic acids is their stability in the presence of organic material. This is in contrast to chlorine, which reacts with organic matter to form chloro-nitrogen compounds that have little antimicrobial activity (Keener, Bashor, Curtis, Sheldon, & Karthariou, 2004). For this reason, organic acids alone or in combination with chlorine can be effective in environments with a large amount of organic material. It is important to consider that suboptimal parameters and low concentrations of acid, resulting in insufficient acidic conditions, may lead to bacterial adaptation and increased resistance to treatment. It is known that *Salmonella*, especially, is tolerant to acidic environments as low as pH 4, and some strains may endure for extended periods in even more acidic environments if they are first allowed to adapt to a moderately low pH (Foster, 1995). Adaptation takes place under similar conditions in other pathogens such as *E. coli* O157:H7 and *L. monocytogenes* (Koutsoumanis & Sofos, 2004). Similar to *S*. Typhimurium, *L. monocytogenes* has also been reported to survive in acidic laboratory environments as low as pH 4 (Phan-Thanh & Montagne, 1998). This potential for development of resistant strains illustrates the need to maintain optimal processing specifications and acid concentrations. These controls will guarantee a sufficient pH and increase the effectiveness of the treatment.

18.4.5 Essential oils

The essential oils of plants have displayed extensive effects against many foodborne pathogens, although they are overall more effective against Gram-positive bacteria. To date, there are over 1340 plants characterized as having antimicrobially active components, and these components currently number more than 30,000. Only a few of the essential oils in this list have been described as useful for commercial application (Tajkarimi, Ibrahim, & Cliver, 2010). A large number of these essential oils have shown activity against pathogens *in vitro*. However *in vitro* efficacy, typically measured as minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC), does not directly predict the effects application in a food matrix (Burt, 2004). Typical components of a food matrix have been shown to interfere with the antimicrobial activity of several essential oils. Gutierrez, Barry-Ryan, and Bourke (2008) found that potato starch and sunflower oil at 5% and 10% in model media could reduce activity of essential oils against *L. monocytogenes*. Oftentimes when added directly into products, essential oils may also contribute to the sensory properties of the product, so discretion should be used

when applying essential oils onto poultry products. Therefore, essential oils are oftentimes used as marinades for poultry products for their bactericidal and bacteriostatic qualities. Some marinades are effective at reducing and/or eliminating low levels of bacterial pathogens, but higher levels of inoculation see little to no reduction (Pathania, McKee, Bilgili, & Singh, 2010). It is important to note that in some cases, the MIC for a particular essential oil may be well above levels that consumers will find palatable; therefore, in order to balance organoleptic and antimicrobial properties, it is necessary to determine an accurate MIC of a particular essential oil for each product to which the oil will be added (Lambert, Skandamis, Coote, & Nychas, 2001). Additionally, the potential to achieve lower MIC values using combinations of essential oils has been suggested. In this application, the dynamics between sensory characteristics desired for the product and each essential oil should be considered as well (Gutierez et al., 2008).

Nazer, Kobilinsky, Tholozan, and Dubois-Brissonnet (2005) documented the inhibitory effect of aromatic compounds like thymol, carvacarol, citral, eugenol, and geraniol in combination with acids on growth of *S*. Typhimurium in laboratory media. Tassou, Drosinos, and Nychas (1995) reported a synergistic effect of pH and mint essential oils in inhibition of *S. enteritidis* and *L. monocytogenes* in food and laboratory media. Mint essential oil at a concentration of as low as 0.4% also has an inhibitory action against *S. enteritidis* and *Staphylococcus aureus* and reduced the total viable counts by 3 and 6−7 logs, respectively (Tassou, Koutsoumanis, & Nychas, 2000). During a simulated chill, a herbal extract combination was able to reduce naturally occurring *Campylobacter* levels over 1.4 $\log_{10}$ CFU/ml lower than carcasses treated with water alone, although quality carcass measurements were not reported (Dickens, Berrang, & Cox, 2000). Reductions of *C. jejuni*, 2.4 $\log_{10}$ CFU/ml after 24 h and below detection after 48 h at 4 °C were reported in a marinade of vegetable oil, water, spice, and NaCl, whereas no changes in the survival of *C. jejuni* were reported on marinated chicken drumsticks and breast fillets (Perko-Makela, Koljonen, Miettinen, & Hanninen, 2000). The shelf life of ready- to-cook (RTC) poultry products was extended to up to 14 days when MAP was combined with 1.5% chitosan and 0.2% thymol treatments (Giatrakou, Ntzimani, & Savvaidis, 2010).

Certain plant products, known as herbs and spices, have shown antimicrobial activity when used in product formulations and can prolong shelf life when used singly or in various combinations. Some of these include oregano, rosemary, thyme, sage, basil, turmeric, ginger, garlic, nutmeg, clove, mace, savory, and fennel. Although these herbs and spices can inhibit many different bacteria, including both Gram-positives and Gram-negatives, their successful application is dependent on storage factors such as pH, temperature, and oxygen levels (Tajkarimi et al., 2010).

18.4.5.1 Turmeric

With its use as the flavor and yellow color in many curry dishes throughout the world, turmeric is also used as a remedy to reduce inflammation and to treat various digestive issues (Chattopadhay, Biswas, Bandyopadhyaya, & Banerjee, 2004). The roots are boiled and then oven dried, which assists in the development of the yellow color.

The powerful antioxidant activity of turmeric comes primarily from curcumin, the active ingredient, which has been shown to have a wide array of activity. Regarding poultry-borne bacteria, turmeric essential oil demonstrated a wide (0.7−10% vol/vol) range of MIC (minimum inhibitory concentration) against *L. monocytogenes* but no effects against *S.* Typhimurium DT104 (Thongson, Davidson, Mahakarnchanakul, & Vibulsresth, 2005). The anti-adhesion properties of extracts have also been investigated as an alternative to clinical antibiotic therapy in humans. The adhesion of bacterial cells to epithelial cells and subsequent invasion is the primary means of human illness development. Turmeric showed a small amount of anti-adhesion activity against *C. jejuni* on HT-29 cells (Bensch et al., 2011).

Inoculated chicken breast fillets marinated with turmeric alone did not show inhibition against multiple strains of *C. jejuni* but did eliminate *S. enteritidis* after 36 h of incubation (Murali, Kumar-Phillips, Rath, Marcy, & Slavik, 2012). Murali et al. (2012) also demonstrated that combinations of green tea extract + turmeric and lemon extract + turmeric were able to reduce *C. jejuni* and *S. enteritidis* after 24 h incubation; however, green tea + turmeric did not affect *S. enteritidis* during the time studied. A combination of lemon extract, green tea extract, and turmeric reduced *C. jejuni* and *S. enteritidis* below detection after only 12 h of incubation. Turmeric pastes did not show any antimicrobial activity against *E. coli* 0157:H7 in both laboratory buffer and inoculated ground beef (Gupta & Ravishankar, 2005).

18.4.5.2 Carvacrol

Carvacrol is a compound found predominantly in the essential oil of oregano (*Origanum glandulosum)* and disintegrates the cell wall of Gram-negative bacteria (Helander et al., 1998). In relation to the quality of poultry products, carvacrol combined with other essential oil compounds and low temperatures was able to enhance the quality of poultry patties (Mastromatteo, Lucera, Sinigaglia, & Corbo, 2009). Lucera, Mastromatteo, Sinigaglia, and Corbo (2009) used carvacrol alone to inhibit the development of lipid oxidation and preserve the instrumental color values of ground poultry patties. A combination of carvacrol, cinnamaldehyde, thymol, and oregano oil was able to inhibit anaerobic *C. perfringens* germination and outgrowth in ground turkey products during abusive temperatures (Juneja & Friedman, 2007). Burt, Fledderman, Haagsman, van Knapen, and Veldhuizen (2007) found that carvacrol vapor was able to significantly impact *S.* Enteritidis inoculated on raw chicken breast meat. A solution of carvacrol and ethanol was applied to a paper disc and incubated with the inoculated chicken within Petri plates. The authors found that around 3.7-log CFU reduction was achieved after incubation for 3 h at 37 °C. The activity of carvacrol is highly dependent on temperature, as experiments have shown reduced activity against both *Salmonella* and *L. monocytogenes* at low temperatures (Burt et al., 2007; Veldhuizen, Creutzberg, Burt, & Haagsman, 2007). This would make treatment difficult under temperatures typical for post-chill facilities.

18.4.5.3 Thyme

The essential oil of thyme has one of the highest antimicrobial activities of the known plant extracts primarily because it contains large amounts of phenols such as thymol,

p-cymene, carvacrol, and γ-terpinene (Giatrakou et al., 2010; Gutierrez, Barry-Ryan, & Bourke, 2009). Although these compounds are generally more effective against Gram-positive bacteria, thymol and carvacrol have displayed the ability to dissolve the lipopolysaccharide (LPS) layer of *E. coli* and *S.* Typhimurium. At low pH, the lipid-solubility of these phenolic compounds increases, emphasizing the necessity to control acidity of the food product to ensure maximum solubility in LPS and therefore maximum antibacterial activity (Holley & Patel, 2005).

Antimicrobial properties of thyme essential oil have greater efficacy with low oxygen concentrations (Paster et al., 1990). This indicates that the incorporation of modified atmosphere packaging (MAP) may enhance the effectiveness of thyme oil. In fact, the application of thyme in RTC poultry products has been evaluated in combination with MAP as well as with chitosan (Giatrakou et al., 2010). The study showed that the combination of thyme and MAP increased the shelf life of RTC chicken-pepper kebabs by 6 days, and the combination of thyme, chitosan, and MAP increased shelf life by 14 days, while also reducing total aerobes, lactic acid bacteria, *Pseudomonas* spp., Enterobacteriaceae, and fungi. Chouliara and Kontominas (2007) also reported 6-day increases in the shelf life of chicken breast meat using a combination of 0.5% thyme oil and MAP as well as a 5-day increase with just 0.5% thyme oil. Additionally, L*, a*, and b* values remained within acceptable parameters after storage. Aureli, Costantini, and Zolea (1992) achieved significant reductions against *L. monocytogenes* in meat products. These characteristics further demonstrate the potential of this additive to enhance product quality.

18.4.5.4 Spice extracts

The extracts of cinnamon, cloves, and other spices are known to have broad-spectrum antimicrobial activity, affecting both Gram-positive and Gram-negative bacteria (Gill & Holley, 2004; Hoque, Inatsu, Juneja, & Kawamoto, 2007). The major bactericidal components of their essential oils are thought to be eugenol in cloves and cinnamaldehyde in cinnamon (Hoque et al., 2007). Both of these phenolic compounds have the potential to inhibit pathogens in food products, as eugenol has shown activity against *E. coli* O157:H7 and *L. monocytogenes*, while cinnamaldehyde demonstrated inhibition of *Clostridium botulinum*, *Staphylococcus aureus*, *E. coli* O157:H7, and *S.* Typhimurium (Blaszyk & Holley, 1998; Bowles, Sackitey, & Williams, 1995; Gill & Holley, 2004; Helander et al., 1998). Conflicting mechanisms of action exist describing antibacterial properties of eugenol and cinnamaldehyde. The leading theory involves disruption of the bacterial cell membrane; however, other theories involving inhibition of various metabolic processes and enzymes have been supported by some studies (Gill & Holley, 2004).

There is existing evidence supporting the usefulness of spice extracts in poultry products. Hoque et al. (2007) reported reduction of *L. monocytogenes* inoculated into ground chicken to undetectable levels after 1 day of exposure to 10% clove essential oil and a reduction of 2-log CFU/g after 1 day of treatment with cinnamon essential oil. Ravishankar, Zhu, Olsen, McHugh, and Friedman (2009) observed between 4.3 and 2.8 log reduction in *S. enterica* and between 5.2 and 1.2 log reduction in *E. coli* O157:H7 for chicken breast wrapped in edible films containing 3% cinnamaldehyde

and stored at two different temperatures (4 and 23 °C). For 1.5% cinnamaldehyde films, they observed >3 to >1 log reductions in both organisms. At 0.5% cinnamaldehyde, reductions could only be achieved at 23 °C (1.6 log reduction for *S. enterica* and 1.8 log reduction for *E. coli* O157:H7).

18.4.5.5 Grapefruit seed extract

Grapefruit seed extract (GSE) has multiple applications in poultry product quality. This extract contains several compounds known for their antioxidant capabilities such as tocopherols, citric acid, and ascorbic acid. For this reason, GSE has been used in combination with other extracts such as thymol and carvacrol to prevent lipid oxidation in ostrich, chicken, and turkey meat (Lucera et al., 2009). In this study, GSE was shown to reduce lipid oxidation, but less so than thymol and carvacrol. Preventing oxidation is important to the shelf life of a product, as it can lead to undesirable changes in flavor due to rancidity, as well as losses in other organoleptic qualities such as color, aroma, and texture. *In vitro* testing of antimicrobial films containing a combination of GSE and EDTA has been promising and has shown strong inhibitory effects against several bacteria including *Listeria innocua*, *E. coli*, *S.* Enteritidis, *Staphyloccocus aureus*, and *Micrococcus luteus* (Cha, Choi, Chinnan, & Park, 2002). Riedel, Brøndsted, Rosenquist, Haxgart, and Christensen (2009) demonstrated a significant reduction in *C. jejuni* on inoculated chicken skin and chicken breast meat dipped in a treatment solution containing GSE. However, the authors noted that this antimicrobial effect could have been caused by synthetic disinfectants, which have been found to contaminate commercial GSE extracts (Riedel et al., 2009; Takeoka, Dao, Wong, Lundin, & Mahoney, 2001).

18.4.5.6 Hop extracts

The female flower of the hop plant, *Humulus lupulus*, is known for its antimicrobial properties and has long been used as an important preservative and flavoring agent of beer. The antimicrobial aspects of hops are primarily attributed to the alpha acids (humulones) and beta acids (lupulones). These acids are also responsible for imparting a bitter flavor to the flower. Active mostly against only Gram-positive bacteria, the mechanism of humulone and lupulone bactericidal ability is by disruption of the cell membrane (De Keukeleire, 2000; Simpson & Smith, 1992; Teuber and Schmealreck, 1973). The FSIS has approved the inclusion of beta acids as antimicrobials for cooked meat and in casings for ready-to-eat (RTE) meat products; however, there are few published studies on the use of these hop-components in poultry products. Shen, Georgnaras, Kendall, and Sofos (2009) reported the use of beta acids to eliminate *L. monocytogenes* from the surface of frankfurters. Frankfurters inoculated with *L. monocytogenes* were dipped in aqueous solutions of beta acids at several concentrations, vacuum packaged, and stored under refrigeration. Levels of *L. monocytogenes* were reduced by1.3−1.6 log CFU after initial dip (compared to 1 log reduction in control dip). Lag-phase was lengthened and growth rates were suppressed during storage. More research is required to determine the efficacy of beta acids on other products as well as the effect of such treatments on sensory qualities.

18.4.5.7 Citrus extracts

The extracts of citrus fruits such as oranges have been shown to have antimicrobial activity against various microorganisms both *in vitro* and in food systems. Dabbah, Edwards, and Moats (1970) investigated the action of various citrus oils and their actions against numerous food-borne bacteria including salmonellae, enterobacteriaceae, *S. aureus*, and *B. subtilis*, as well as pseudomonads and other food spoilage organisms. Gram-positive organisms were more susceptible to essential oils—and terpeneless fractions of these oils had more activity overall. In this same study, orange oil and terpineol considerably lengthened the shelf life of pasteurized milk. O'Bryan, Crandall, Chalova, and Ricke (2008) showed antimicrobial activity of orange essence terpenes against 11 *Salmonella* strains, reporting MICs between 0.125 and 0.5%. In a disk diffusion assay, commercial orange oil fractions were shown to have similar inhibitory effects against both ciprofloxacin-resistant and ciprofloxacin-sensitive *C. jejuni* (Nannapaneni et al., 2009). The potential for using citrus extracts to control food-borne microbes in poultry products was shown by Valtierra-Rodríguez, Heredia, García, and Sánchez (2010) when lime, plum, and sour orange peel extracts were able to eliminate detectible *Campylobacter* from inoculated chicken skin. However, Fisher and Phillips (2006) observed reduced ability of lemon, orange and bergamot essential oils to inhibit *C. jejuni*, *E. coli* O157, *L. monocytogenes*, *Bacillus cereus*, and *S. aureus* on inoculated chicken skin compared to disk diffusion methods.

18.4.6 Cultured sugar

The use of marinades for added value poultry products not only provides enhanced organoleptic qualities but also provides opportunities for extending the shelf life of certain products. Marination has been investigated as a strategy to enhance the quality and safety of poultry products under storage conditions (Carroll & Alvarado, 2008; Smith & Acton, 2001). In addition to marinades utilizing lactic acid and/or salt, the use of cultured sugar has been reported as a comparable alternative in terms of food safety and quality. Cultured sugar/vinegar blends (CSV) are produced by fermentation using certain cultures, resulting in a complex product containing sugars, organic acids, peptides, and aromatics (Park, Hong, & Yoon, 2014). In a shelf-life study by McDonnell, Glass, and Sindelar (2013), the use of CSV in boneless ham and deli-style turkey breast was able to delay propagation of *L. monocytogenes* by an additional 4 weeks longer than the control, but efficacy was improved further by addition of nitrites. According to Park et al. (2014), inclusion of 3% CSV with precooked chicken breast showed increased inhibition of *C. jejuni* and *S.* Typhimurium over controls. Additionally, treating with CSV resulted in improved texture for both frozen and RTE chicken breasts; this information supports the use of CSV to enhance both the safety and quality of further processed poultry products.

18.4.7 Bacteriophages

Bacteriophages, also known as phages, have the ability to infect bacteria and most bacterial species have their own unique bacteriophages (Loc Carrillo et al., 2005).

Adoption of phage therapy has proven to be effective (Sulakvelidze, Alavidze, & Morris, 2001); however, widespread use has not been adopted in the poultry industry. In addition to applications on live broilers, the application of lytic bacteriophage mixtures is permitted on RTE meat and poultry products to control *L. monocytogenes* (USDA, 2012).

Reducing the bacteria shed by broilers before they enter a commercial processing facility is the most ideal approach to meeting USDA-FSIS performance standards. One such approach involves the use of lytic bacteriophages to reduce the gut colonization of poultry-borne pathogens. Loc Carrillo et al. (2005) and Wagenaar, Van Bergen, Mueller, Wassenaar, and Carlton (2005) separately demonstrated that in an experimental situation this methodology is able to achieve between 2 and 3 $\log_{10}$ reductions in *Campylobacter* shedding. Additionally, *Salmonella* spp. recovery on poultry carcasses was reduced significantly using a *Salmonella* specific bacteriophage (Higgins et al., 2005). There are concerns that bacteriophage-resistant *Campylobacter* and *Salmonella* spp. will be selected for and be more difficult to eliminate using chemical decontamination methods in the processing plant environment.

18.4.8 Bacteriocins

Bacteriocin proteins are bacterial metabolites with antimicrobial properties against other species of bacteria. One of the most extensively studied bacteriocin is nisin, produced by *Lacobacillus lactis* (Bolder, 1997). These proteins are normally found in meat, as *Lactobacillus* is a common member of natural microflora in this food matrix. Therefore, consumption of these compounds is assumed to be safe (Yang & Ray, 1994). In fact, nisin has been granted GRAS status by the World Health Organization, confirming its non-toxic activity (Abee, Krockel, & Hill, 1995). There are two main applications of bacteriocin for the inhibition of unwanted bacteria in a food product. The first is direct addition of the bacteriocin to the product and the second method involves inoculating the product with a live culture of bacteriocin-producing bacteria (Bolder, 1997). Nisin's mode of action involves pore formation in the cell membrane of Gram-positive bacteria, resulting in cell lysis. These pores are selective for potassium ions and inorganic phosphate (Van Schaik, Gahan, & Hill, 1999). As antimicrobial activity of nisin is not specific to Gram-negative bacteria, the use of additional components is needed to prevent attachment of pathogens like *Salmonella* and *E. coli*. The inclusion of 20 mM EDTA or citric acid monohydrate with 50−100 μg/ml nisin was able to produce antimicrobial activity against *Salmonella* (Stevens, Sheldon, Klapes, & Klaenhammer, 1992).

18.5 Conclusion and future trends

Successful control of pathogens requires a truly integrated approach all the way from the hatcheries to the processing plant. To this day, given the intensive and extensive research that has been documented and ongoing, there is no single critical control point that has been identified to ensure pathogen reduction in the poultry production

continuum. It is clearly evident that a successful pathogen control program entails effective on-farm practices such as treatment of drinking water, following adequate food withdrawal programs, and treatment of litter in order to reduce the microbial load on live birds entering the processing plants. However, the control strategies need to continue into the processing plants, where HACCP and adequate prerequisite programs in addition to antimicrobials should be effectively used and incorporated as part of an overall food safety program. While natural antimicrobials are popularly being used and marketed in the poultry industry, they should never be considered as substitutes for good manufacturing practices and proper handling and storage of poultry meat and its products.

References

Abee, T., Krockel, L., & Hill, C. (1995). Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *International Journal of Food Microbiology*, *28*, 169−185.

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*, *66*(5), 2001−2005.

Al-Chalaby, Z. A. M., Hinton, M., & Linton, A. H. (1985). Failure of drinking water sanitization to reduce the incidence of natural *Salmonella* in broiler chickens. *Veterinary Record*, *116*, 364−365.

Aureli, P., Costantini, A., & Zolea, S. (1992). Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *Journal of Food Protection*, *55*(5), 344−348.

Bailey, J. S., Blankenship, L. C., & Cox, N. A. (1991). Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. *Poultry Science*, *70*, 2433−2438.

Bashor, M. P., Curtis, P. A., Kenner, K. M., Sheldon, B. W., Kathariou, S., & Osborne, J. A. (2004). Effect of carcass washers on *Campylobacter* contamination in large broiler processing plants. *Poultry Science*, *83*, 1232−1239.

Batz, M. B., Hoffman, S., & Glenn-Morris, J. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitations. *Journal of Food Protection*, *75*(7), 1278−1291.

Behnsen, J., Deriu, E., Sassone-Corsi, M., & Raffatellu, M. (2013). Probiotics: properties, examples, and specific applications. *Cold Spring Harbor Perspectives in Medicine*, *3*(3), a010074. Available from http://perspectivesinmedicine.org/content/3/3/a010074.full.pdf+html.

Bensch, K., Tiralongo, J., Schmidt, K., Matthias, A., Bone, K. M., Lehmann, R., et al. (2011). Investigations into the antiadhesive activity of herbal extracts against *Campylobacter jejuni*. *Phytotherapy Research*, *25*, 1125−1132.

Bilgili, S. F., Waldroup, A. L., Zelenka, D., & Marion, J. E. (2002). Visible ingesta on prechill carcasses does not affect the microbiological quality of broiler carcasses after immersion chilling. *Journal of Applied Poultry Research*, *11*, 233−238.

Bilgili, S. F., Conner, D. E., Pinion, J. L., & Tamblyn, K. C. (1998). Broiler skin color as affected by organic acids: influence of concentration and method of application. *Poultry Science*, *77*(5), 752−757.

Blaszyk, M., & Holley, R. A. (1998). Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. *International Journal of Food Microbiology*, *39*, 175−183.

Bolder, N. M. (1997). Decontamination of meat and poultry carcasses. *Trends in Food Science and Technology*, *8*, 221–227.

Bowles, B. L., Sackitey, S. K., & Williams, A. C. (1995). Inhibitory effects of flavor compounds on *Staphyloccocus aureus* WRRC B124. *Journal of Food Safety*, *15*, 3370347.

Brenes, A., & Roura, E. (2010). Essential oils in poultry nutrition: main effects and modes of action. *Animal Feed Science and Technology*, *158*(1–2), 1–14.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223–253.

Burt, S. A., Fledderman, M. J., Haagsman, H. P., van Knapen, F., & Veldhuizen, E. J. A. (2007). Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour. *International Journal of Food Microbiology*, *119*(3), 346–350.

Burton, C. H., Cumby, T. R., & Tinker, D. B. (2004). Treatment and disposal of poultry processing waste. In G. C. Mead (Ed.), *Poultry meat processing and quality* (pp. 345–376). CRC, Woodhead.

Callaway, T. R., Edrington, T. S., Anderson, R. C., Harvey, R. B., Genovese, K. J., Kennedy, C. N., et al. (2008). Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Animal Health Research Reviews*, *9*(2), 217–225.

Carroll, C. D., & Alvarado, C. Z. (2008). Comparison of air and immersion chilling on meat quality and shelf life of marinated broiler breast fillets. *Poultry Science*, *87*, 368–372.

Cha, D. S., Choi, J. H., Chinnan, M. S., & Park, H. J. (2002). Antimicrobial films based on Na-alginate and κ-carrageenan. *Lwt-food Science and Technology*, *35*(8), 715–719.

Chattopadhay, I., Biswas, K., Bandyopadhyaya, U., & Banerjee, R. K. (2004). Turmeric and curcumin: biological actions and medicinal applications. *Current Science*, *87*(1), 44–53.

Chaveerach, P., Lipman, L. J., & van Knapen, F. (2004). Antagonistic activities of several bacteria on in vitro growth of 10 strains of *Campylobacter jejuni/coli*. *International Journal of Food Microbiology*, *90*, 43–50.

Chouliara, E., & Kontominas, M. G. (2007). Combined effect of thyme essential oil and modified atmosphere packaging to extend shelf-life of fresh chicken meat. In V. K. Singh, J. N. Govil, K. Ahmad, & R. K. Sharma (Eds.), *Natural products* (pp. 423–441). India: Studium Press.

Collins, M. D., & Gibson, G. R. (1999). Probiotics, prebiotic, and synbiotics: approaches for modulating the microbial ecology of the gut. *American Journal of Clinical Nutrition*, *69*(5), 1052s–1057s.

Conner, D. E., Davis, M. A., & Zhang, L. (2001). Poultry-borne pathogens: plant considerations. In A. R. Sams (Ed.), *Poultry meat processing* (pp. 137–158). Boca Raton, FL: CRC Press.

Corry, J. E., James, S. J., Purnell, G., Barbedo-Pinto, C. S., Chochois, Y., Howell, M., et al. (2007). Surface pasteurisation of chicken carcasses using hot water. *Journal of Food Engineering*, *79*(3), 913–919.

Cosansu, S., & Ayhan, K. (2012). Effects of lactic and acetic acid on survival of *Salmonella enteritidis* during refrigerated and frozen storage of chicken meats. *Food and Bioprocess Technology*, *5*(1), 372–377.

Cox, N. A., Mercuri, A. J., Juven, B. J., Thomson, J. E., & Chew, V. (1974). Evaluation of succinic acid and heat to improve microbiological quality of poultry meat. *Journal of Food Science*, *39*, 985.

Dabbah, R., Edwards, V. M., & Moats, W. A. (1970). Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Applied Microbiology*, *19*(1), 27–31.

De Keukeleire, D. (2000). Fundamentals of beer and hop chemistry. *Química Nova*, *23*, 108–112.

de los Santos, F. S., Donoghue, A. M., Venkitanarayanan, K., Dirain, M. L., Reyes-Herrera, I., Blore, P. J., et al. (2008). Caprylic acid supplemented in feed reduces enteric *Campylobacter jejuni* colonization in ten-day-old broiler chickens. *Poultry Science*, *87*, 800−804.

de los Santos, F. S., Donoghue, A. M., Venkitanarayanan, K., Metcalf, J. H., Reyes-Herrera, I., Dirain, M. L., et al. (2009). The natural feed additive caprylic acid decreases *Campylobacter jejuni* colonization in market-aged broiler chickens. *Poultry Science*, *88*, 61−64.

Dickens, J. A., Berrang, M. E., & Cox, N. A. (2000). Efficacy of an herbal extract on the microbiological quality of broiler carcasses during a simulated chill. *Poultry Science*, *79*, 1200−1203.

Dickens, J. A., & Whittemore, A. D. (1997). Effects of acetic acid and hydrogen peroxide application during defeathering on the microbiological quality of broiler carcasses prior to evisceration. *Poultry Science*, *76*, 657−660.

Dickens, J. A., & Whittemore, A. D. (1994). Effect of acetic acid and air injection on appearance, moisture pick-up, microbiological quality, and *Salmonella* incidence on processed poultry carcasses. *Poultry Science*, *73*, 582−586.

Dickson, J. S., & Anderson, M. E. (1992). Microbiological decontamination of food animal carcasses by washing and sanitizing systems: a review. *Journal of Food Protection*, *55*, 133−140.

Doores, S. (2005). Organic acids. In P. M. Davidson, J. N. Sofos, & A. L. Brannen (Eds.), *Antimicrobials in foods* (3rd ed.). (pp. 91−142). CRC, Taylor and Francis.

Edens, F. W. (2003). An alternative for antibiotic use in poultry: probiotics. *Revista Brasileira de Ciencia Avicola*, *5*(2), 75−97.

FDA. (2013). *Guidance for Industry: frequently asked questions about GRAS*. December 2004. Available from http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm061846.htm.

Farag, R. S., Daw, Z. Y., Hewedi, F. M., & El-Baroty, G. S. A. (1989). Antimicrobial activity of some Egyptian spice essential oils. *Journal of Food Protection*, *52*, 665−667.

Fisher, K., & Phillips, C. A. (2006). The effect of lemon, orange, and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems. *Journal of Applied Microbiology*, *101*, 1232−1240.

Foster, J. W. (1995). Low pH adaptation and the acid tolerance response of *Salmonella* Typhimurium. *Critical Reviews in Microbiology*, *21*(4), 215−237.

Friedman, M., Henika, P. R., & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection*, *65*, 1545−1560.

Gaggía, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology*, *141*(Suppl.), 15−28.

Giatrakou, V., Ntzimani, A., & Savvaidis, I. N. (2010). Combined chitosan-thyme treatments with modified atmosphere packaging on a ready-to-cook poultry product. *Journal of Food Protection*, *73*(4), 663−669.

Gill, A. O., & Holley, R. A. (2004). Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Applied and Environmental Microbiology*, *70*(10), 5750−5755.

Gunal, M., Yayli, G., Kaya, O., Karahan, N., & Sulak, O. (2006). The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers. *International Journal of Poultry Science*, *5*(2), 149−155.

Gupta, S., & Ravishankar, S. (2005). A comparison of the antimicrobial activity of garlic, ginger, carrot, and turmeric pastes against *Escherichia coli* O157:H7 in laboratory buffer and ground beef. *Foodborne Pathogens and Disease*, *2*(4), 330–340.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, *124*(1), 91–97.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. *Food Microbiology*, *26*(2), 142–150.

Griggs, J. P., & Jacob, J. P. (2005). Alternatives to antibiotics for organic poultry production. *Journal of Applied Poultry Research*, *14*, 750–756.

Hajati, H., & Rezaei, M. (2010). The application of prebiotics in poultry production. *International Journal of Poultry Science*, *9*(3), 298–304.

Helander, I. M., Alakomi, H. L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., et al. (1998). Characterization of the action of selected essential oil components on gram-negative bacteria. *Journal of Agricultural and Food Chemistry*, *46*(9), 3590–3595.

Higgins, J. P., Higgins, S. E., Guenther, K. L., Huff, W., Donoghue, A. M., Donoghue, D. J., et al. (2005). Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. *Poultry Science*, *84*, 1141–1145.

Higgins, J. P., Higgins, S. E., Vicente, J. L., Wolfenden, A. D., Tellex, G., & Hargis, B. M. (2007). Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poultry Science*, *86*(8), 1662–1666.

Hinton, A., Jr., Cason, J. A., Buhr, R. J., & Liljebjelke, K. (2009). Bacteria recovered from whole-carcass rinsates of broiler carcasses washed in a spray cabinet with lauric acid-potassium hydroxide. *International Journal of Poultry Science*, *8*(11), 1022–1027.

Hinton, M., & Linton, A. H. (1988). Control of salmonella infections in broiler chickens by the acid treatment of their feed. *Veterinary Record*, *123*, 416–421.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*, *22*(4), 273–292.

Hoque, M. M., Inatsu, M. B., Juneja, V., & Kawamoto, S. (2007). Antimicrobial activity of cloves and cinnamon extracts against food borne pathogens and spoilage bacteria, and inactivation of *Listeria monocytogenes* in ground chicken meat with their essential oils. *Journal of Food Science and Technology*, *72*, 9–21.

Hwang, C. A., & Beuchat, L. R. (1995). Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin. *Journal of Food Protection*, *58*(1), 19–23.

Izat, A. L., Colberg, M., Thomas, R. A., Adams, M. H., & Driggers, C. D. (1990). Effects of lactic acid in processing waters on the incidence of *Salmonellae* on broilers. *Journal of Food Quality*, *13*(4), 295–306.

Jimenez, S. M., Salsi, M. S., Tiburzi, M. C., Rafaghelli, R. C., & Pirovani, M. E. (1999). Combined use of acetic acid treatment and modified atmosphere packaging for extending the shelf-life of chilled chicken breast portions. *Journal of Applied Microbiology*, *87*(3), 339–344.

Jackson, W. C., Curtis, P. A., Carawan, R. E., Keener, K. M., & Taylor, M. C. (1999). *Survey shows that poultry processors can save money by conserving water*. No. CD-23. Raleigh, NC: North Carolina Cooperative Extension Service, NC State University.

Juneja, V. K., & Friedman, M. (2007). Carvacrol, cinnamaldehyde, oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. *Journal of Food Protection*, *70*(1), 218–222.

Kabir, S. M. (2009). The role of probiotics in the poultry industry. *International Journal of Molecular Sciences*, *10*, 3531−3546.

Karapinar, M., & Aktuğ, Ş. E. (1987). Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. *International Journal of Food Microbiology*, *4*, 161−166.

Keener, K. M., Bashor, M. P., Curtis, P. A., Sheldon, B. W., & Kathariou, S. (2004). Comprehensive review of *Campylobacter* and poultry processing. *Comprehensive Reviews in Food Science and Food Safety*, *3*, 105−116.

Koutsoumanis, K. P., & Sofos, J. N. (2004). Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium after habituation at different pH conditions. *Letters in Applied Microbiology*, *38*(4), 321−326.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J., & Nychas, G. J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, *91*(3), 453−462.

La Ragione, R. M., & Woodward, M. J. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Veterinary Microbiology*, *94*(3), 245−256.

Loc Carrillo, C., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Applied and Environmental Microbiology*, *71*, 6554−6563.

Lucera, A., Mastromatteo, M., Sinigaglia, M., & Corbo, M. R. (2009). Combined effects of thymol, carvacrol and grapefruit seed extract on lipid oxidation and colour stability of poultry meat preparations. *International Journal of Food Microbiology*, *44*(11), 2256−2267.

Mani-López, E., García, H. S., & López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, *45*(2), 713−721.

Mastromatteo, M., Lucera, A., Sinigaglia, M., & Corbo, M. R. (2009). Combined effects of thymol, carvacrol and temperature on the quality of non conventional poultry patties. *Meat Science*, *83*(2), 246−254.

McDonnell, L. M., Glass, K. A., & Sindelar, J. J. (2013). Identifying ingredients that delay outgrowth of *Listeria monocytogenes*in natural, organic, and clean-label ready-to-eat meat and poultry products. *Journal of Food Protection*, *8*, 1308−1479.

Metcalf, J. H., Donoghue, A. M., Venkitanarayanan, K., Reyes-Herrera, I., Aguiar, V. F., Blore, P. J., et al. (2011). Water administration of the medium-chain fatty acid caprylic acid produced variable efficacy against enteric *Campylobacter* colonization in broilers. *Poultry Science*, *90*, 494−497.

Milillo, S. R., Martin, E., Muthaiyan, A., & Ricke, S. C. (2011). Immediate reduction of *Salmonella enterica* serotype Typhimurium viability vie membrane destabilization following exposure to multiple-hurdle treatments with heated, acidified organic acid salt solutions. *Applied and Environmental Microbiology*, *77*(11), 3765−3772.

Morishita, T. Y., Aye, P. P., Harr, B. S., Cobb, C. W., & Clifford, J. R. (1997). Evaluation of an avian-specific probiotic to reduce the colonization and shedding of *Campylobacter jejuni* in broilers. *Avian Diseases*, *41*, 850−855.

Murali, N., Kumar-Phillips, G. S., Rath, N. C., Marcy, J., & Slavik, M. F. (2012). Antibacterial activity of plant extracts of foodborne bacterial pathogens and food spoilage bacteria. *Agriculture, Food and Analytical Bacteriology*, *2*, 209−221.

Nannapaneni, R., Chalova, V. I., Story, R., Wiggins, K. C., Crandall, P. G., Ricke, S. C., et al. (2009). Ciprofloxacin-sensitive and ciprofloxacin-resistance *Campylobacter jejuni* are

equally susceptible to natural orange oil-based antimicrobials. *Journal of Environmental Science and Health*, *44*(6), 571−577.

Nazer, A. I., Kobilinsky, A., Tholozan, J. L., & Dubois-Brissonnet, F. (2005). Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* Typhimurium: a synergistic effect? *Food Microbiology*, *22*, 391−398.

Northcutt, J. K., & Jones, D. R. (2004). A survey of water use and common industry practices in commercial broiler processing facilities. *Journal of Applied Poultry Research*, *13*, 48−54.

Nourmohammadi, R., Hosseini, S. M., & Farhangfar, H. (2010). Influence of citric acid and microbial phytase on growth performance and carcass characteristics of broiler chickens. *American Journal of Animal and Veterinary Sciences*, *5*(4), 282−288.

O'Bryan, C. A., Crandall, P. G., Chalova, V. I., & Ricke, S. C. (2008). Orange essential oils antimicrobial activities against *Salmonella* spp. *Journal of Food Science*, *73*(6), M264−M267.

Okolocha, E. C., & Ellerbroek, L. (2005). The influence of acid and alkaline treatments on pathogens and the shelf life of poultry meat. *Food Control*, *16*(3), 217−225.

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., et al. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998−2008. *Emerging Infectious Diseases*, *19*, 407−415.

Park, N. Y., Hong, S. H., & Yoon, K. S. (2014). Effects of commercial marinade seasoning and a natural blend of cultured sugar and vinegar on *Campylobacter jejuni* and *Salmonella* Typhimurium and the texture of chicken breasts. *Poultry Science*, *93*, 719−727.

Paster, N., Juven, B. J., Shaaya, E., Menasherov, M., Nitzan, R., Weisslowicz, H., et al. (1990). Inhibitory effect of oregano and thyme essential oils on moulds and foodborne bacteria. *Letters in Applied Microbiology*, *11*, 33−37.

Pathania, A., McKee, S. R., Bilgili, S. F., & Singh, M. (2010). Antimicrobial activity of commercial marinades against multiple strains of *Salmonella* spp. *International Journal of. Food Microbiology*, *139*(3), 214−217.

Patrick, T. E., Goodwin, T. L., Collins, J. A., Wyche, R. C., & Love, B. E. (1972). Steam versus hot-water scalding in reducing bacterial loads on the skin of commercially processed poultry. *Applied Microbiology*, *23*(4), 796−798.

Perko-Makela, P., Koljonen, M., Miettinen, M., & Hanninen, M. L. (2000). Survival of *C. jejuni* in marinated and non-marinated chicken products. *Journal of Food Safety*, *20*, 209−216.

Phan-Thanh, L., & Montagne, A. (1998). Physiological and biochemical aspects of the acid survival of *Listeria monocytogenes*. *Journal of General and Applied Microbiology*, *44*, 183−191.

Ravishankar, S., Zhu, L., Olsen, C. W., McHugh, T. H., & Friedman, M. (2009). Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens on meat and poultry products. *Journal of Food Science*, *74*(8), M440−M445.

Restaino, L., Frampton, E. W., Bluestain, R. L., Hemphill, J. B., & Regutti, R. R. (1994). Antimicrobial activity of new organic acid anionic surfactants against various bacterial strains. *Journal of Food Protection*, *57*, 496−501.

Revolledo, L., Ferreira, A. J. P., & Mead, G. C. (2006). Prospects in *Salmonella* control: competitive exclusion, probiotics, and enhancement of avian intestinal immunity. *Journal of Applied Poultry Research*, *15*, 341−351.

Riedel, C. T., Brøndsted, L., Rosenquist, H., Haxgart, S. N., & Christensen, B. B. (2009). Chemical decontamination of *Campylobacter jejuni* on chicken skin and meat. *Journal of Food Protection*, *72*(6), 1173−1180.

Ross, Z. M., O'Gara, E. A., Hill, D. J., Sleightholme, H. V., & Maslin, D. J. (2001). Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Applied and Environmental Microbiology*, *67*, 475−480.

Russell, S. M. (2012). Risk assessment of *Salmonella* from poultry sources. In *Controlling* Salmonella *in poultry production and processing* (pp. 15−34). CRC, Taylor and Francis.

Sammel, L. M., & Claus, J. R. (2003). Citric acid and sodium citrate effects on reducing pink color defect of cooked intact turkey breasts and ground turkey rolls. *Journal of Food Science*, *68*(3), 874−878.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *International Journal of Food Microbiology*, *141*(Suppl.), 98−108.

Schneitz, C., Hakkinen, M., Nuotio, L., Nurmi, E., & Mead, G. (1990). Droplet application for protecting chicks against *Salmonella* colonization by competitive exclusion. *Veterinary Record*, *126*(20), 510.

Seifert, S., & Watzl, B. (2007). Inulin and oligofructose: review of experimental data on immune modulation. *Journal of Nutrition*, *137*(11), 2563S−2567S.

Simpson, W. J., & Smith, A. R. (1992). Factors affecting antibacterial activity of hop compounds and their derivatives. *Journal of Applied Bacteriology*, *72*, 327−334.

Sirsat, S. A., Muthaiyan, A., & Ricke, S. C. (2009). Antimicrobials for pathogen reduction in organic and natural poultry production. *Journal of Applied Poultry Research*, *18*, 379−388.

Smith, D. P., & Acton, J. C. (2001). Marination, cooking, and curing of poultry products. In A. R. Sams (Ed.), *Poultry meat processing* (pp. 257−281). Boca Raton, FL: CRC Press.

Sofos, J. N. (2008). Challenges to meat safety in the 21st century. *Meat Science*, *78*, 3−13.

Shen, C., Geornaras, I., Kendall, P. A., & Sofos, J. N. (2009). Control of *Listeria monocytogenes* on frankfurters by dipping in hops beta acids solutions. *Journal of Food Protection*, *72*(4), 702−706.

Stevens, K. A., Sheldon, B. W., Klapes, N. A., & Klaenhammer, T. R. (1992). Effect of treatment conditions on nisin inactivation of gram-negative bacteria. *Journal of Food Protection*, *55*, 763−766.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G. (2001). Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy*, *45*, 649−659.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, *21*, 1199−1218.

Takeoka, G., Dao, L., Wong, R. Y., Lundin, R., & Mahoney, N. (2001). Identification of benzethonium chloride in commercial grapefruit seed extracts. *Journal of Agricultural and Food Chemistry*, *49*(7), 3316−3320.

Tamblyn, K. C., & Conner, D. E. (1997a). Bactericidal activity of organic acids in combination with transdermal compounds against *Salmonella* Typhimurium attached to broiler skin. *Food Microbiology*, *14*, 477−484.

Tamblyn, K. C., & Conner, D. E. (1997b). Bactericidal activity of organic acids against *Salmonella* Typhimurium attached to broiler chicken skin. *Journal of Food Protection*, *6*, 629−633.

Tassou, C., Koutsoumanis, K., & Nychas, G.-J. E. (2000). Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Research International*, *33*(3−4), 273−280.

Tassou, C. C., Drosinos, E. H., & Nychas, G. J. E. (1995). Effects of essential oils from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4 °C and 10 °C. *Journal of Applied Bacteriology*, *78*, 593−600.

Tellez, G., Pixley, C., Wolfenden, R. E., Layton, S. L., & Hargis, B. M. (2012). Probiotics/direct fed microbials for *Salmonella* control in poultry. *Food Research International*, *45*, 628–633.

Teuber, M., & Schmalreck, A. F. (1974). Membrane leakage in *Bacillus subtilis* 168 induced by hop constituents lupulone, humulone, isohumulone and humulinic acid. *Archives of Microbiology*, *94*, 159–171.

Thompson, J. L., & Hinton, M. (1997). Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. *British Poultry Science*, *38*, 59–65.

Thomson, J. E., Banwart, G. J., Sanders, D. H., & Mercuri, A. J. (1967). Effect of chlorine, antibiotics, β-propiolactone, acids, and washing on *Salmonella* Typhimurium on eviscerated fryer chickens. *Poultry Science*, *46*, 146.

Thongson, C., Davidson, P. M., Mahakarnchanakul, W., & Vibulsresth, P. (2005). Antimicrobial effect of Thai spices against *Listeria monocytogenes* and *Salmonella* Typhimurium DT104. *Journal of Food Protection*, *68*(10), 2054–2058.

USDA-Food Safety and Inspection Service. (1996). Pathogen reduction; hazard analysis and critical control Point (HACCP) systems. *Final Rule*, *61*(144).

USDA-Food Safety and Inspection Service. (2006). Product labeling: definition of the term "natural". *Federal Register*, *71*(233).

USDA-Food Safety and Inspection Service. (2008). Temperatures and chilling and freezing procedures. *Federal Register*, 9(381.66).

USDA-Food Safety and Inspection Service. (2009). *New performance standards for* Salmonella *and* Campylobacter *in young chicken and turkey slaughter establishments: Response to comments and announcement of implementation schedule*. Available from http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/2009-0029.pdf.

USDA-Food Safety and Inspection Service. (2012). *Safe and suitable ingredients used in the production of meat, poultry, and egg products*. Available from http://www.fsis.usda.gov/oppde/rdad/fsisdirectives/7120.1.pdf.

USDA-Food Safety and Inspection Service. (2013). *Meat and poultry labeling terms*. Available from http://www.fsis.usda.gov/FactSheets/Meat_&_Poultry_Labeling_Terms/index.asp.

Valtierra-Rodríguez, D., Heredia, N. L., García, S., & Sánchez, E. (2010). Reduction of *Campylobacter jejuni* and *Campylobacter coli* in poultry skin by fruit extracts. *Journal of Food Protection*, *73*(3), 477–482.

Van Schaik, W., Gahan, C. G., & Hill, C. (1999). Acid-adapted *Listeria monocytogenes* displays enhanced tolerance against the lantibiotics nisin and lacticin 3147. *Journal of Food Protection*, *62*(5), 536–540.

Veldhuizen, E. J. A., Creutzberg, T. O., Burt, S. A., & Haagsman, H. P. (2007). Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. *Journal of Food Protection*, *70*(9), 2127–2132.

Vicente, J. L., Torres-Rodriguez, A., Higgins, S. E., Pixley, C., Tellez, G., Donoghue, A. M., et al. (2008). Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Diseases*, *52*(1), 143–146.

Wagenaar, J. A., Van Bergen, M. A. P., Mueller, M. A., Wassenaar, T. M., & Carlton, R. M. (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Veterinary Microbiology*, *109*, 275–283.

Whyte, P., McGill, K., & Collins, J. D. (2003). An assessment of steam pasteurization and hot water immersion treatments for the microbiological decontamination of broiler carcasses. *Food Microbiology*, *20*, 111–117.

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*, E140–E148.

Wolfenden, A. D., Pixley, C. M., Higgins, J. P., Higgins, S. E., Hargis, B. M., Tellez, G., et al. (2007). Evaluation of spray application of a *Lactobacillus*- based probiotic on *Salmonella enteritidis* colonization in broiler chickens. *International Journal of Poultry Science*, *6*(7), 493–496.

Xu, Z. R., Hu, C. H., Xia, M. S., Zhan, X. A., & Wang, M. Q. (2003). Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora, and morphology of male broilers. *Poultry Science*, *82*, 1030–1036.

Yang, R., & Ray, R. (1994). Prevalence and biological control of bacteriocin-producing Psychrotrophic *Leuconostoc* associated with spoilage of vacuum-packed processed meats. *Journal of Food Protection*, *57*, 209–217.

Yang, Z., Li, Y., & Slavik, M. (1998). Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. *Journal of Food Protection*, *61*(7), 829–832.

Zhao, T., Zhao, P., & Doyle, M. P. (2009). Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on lettuce and poultry skin by combinations of levulinic acid and sodium dodecyl sulfate. *Journal of Food Protection*, *72*(5), 928–936.

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