USE OF CHITOSAN-COATED PLASTIC FILMS INCORPORATING ANTIMICROBIALS TO CONTROL THE GROWTH OF *LISTERIA MONOCYTOGENES* ON HAM STEAKS AND COLD-SMOKED SALMON

by

Mu Ye

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science with a major in Food Science

Spring 2008

USE OF CHITOSAN-COATED PLASTIC FILMS INCORPORATING ANTIMICROBIALS TO CONTROL THE GROWTH OF *LISTERIA MONOCYTOGENES* ON HAM STEAKS AND COLD-SMOKED SALMON

by

Mu Ye

Approved: _____
Haiqiang Chen, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Jack Gelb, Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____
Robin Morgan, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Carolyn A. Thoroughgood, Ph.D.
Vice Provost for Research and Graduate Studies

ACKNOWLEDGMENTS

I am thankful to my advisor Dr. Haiqiang Chen for all his support and guidance during the course of my degree. I am also thankful to Dr. Dallas G. Hoover and Dr. Rolf Joerger for their help, advice, and for serving as members of my committee. A sincere appreciation is extended to Hudaa Neetoo and Doris Hicks for all their assistance.

I also want to thank all the colleagues in the Department of Animal & Food Sciences as well as my family back in China for their support throughout the degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Contamination of ready-to-eat food products such as ham steaks and cold-smoked salmon with *Listeria monocytogenes* is a safety concern for the food processing industry. The overall objective of this study was to develop effective antimicrobial packaging films for control of *L. monocytogenes* on cold-smoked salmon and ham steaks. In an aqueous system of culture medium, chitosan-coated plastic films inhibited growth of *L. monocytogenes* in a concentration-dependent manner; however, chitosan-coated plastic films were not able to control the growth of *L. monocytogenes* on ham steaks and cold-smoked salmon. Therefore, five Generally Recognized as Safe (GRAS) antimicrobials were incorporated into chitosan-coated plastic films to enhance antilisterial effectiveness. Incorporating antimicrobials into chitosan-coated plastic films slowed or inhibited the growth of *L. monocytogenes*. The chitosan-coated plastic film containing 10 mg/cm^2 of sodium lactate (SL) was the most effective antimicrobial film on ham steaks and showed excellent long-term antilisterial effect during 12-week storage at 4°C. Chitosan-coated plastic films with 4.5 mg/cm^2 of SL showed complete inhibition of *L. monocytogenes* on smoked salmon during 8-week storage at 4°C. Therefore, chitosan-coated plastic films containing SL could be used to control *L. monocytogenes* on ham steaks and cold-smoked salmon.

Chapter 1

INTRODUCTION

Contamination of *Listeria monocytogenes* is a major safety concern for ready-to-eat (RTE) foods. This ubiquitous bacterium can grow at a temperature range of 1 to 45 °C, over a wide pH range of 4.1 to around 9.6, and at high salt concentrations up to 10% (Shahamat et al., 1980). This organism is a causative agent of listeriosis, a severe invasive illness in humans with a high fatality rate. *L. monocytogenes* has been incriminated in numerous food-borne illness outbreaks associated with RTE foods. In 1998 and 1999, a significant outbreak occurred with frankfurters, which resulted in 21 deaths and approximately 100 reported cases of listeriosis (CDC, 1999). The most recent outbreak occurred in the northeastern United States in 2002, resulting in 10 deaths from the consumption of sliced turkey deli meat (CDC, 2002). The food industry in the United States is currently under a "zero tolerance" policy for *L. monocytogenes* in RTE foods by both USDA Food Safety and Inspection Service (FSIS) and Center for Food Safety and Applied Nutrition (CFSAN), because the infective dose of listeriosis is unknown.

The growth of *L. monocytogenes* on refrigerated, RTE food products causes a serious potential food safety hazard. Many of these choices involve cooked meat products (cooked ham, bologna, galantine, poultry cold cuts, etc) and seafood

(smoked fish, mussel, cooked shrimp, etc), which are prepared in small portions (slices, steaks, small pieces, etc.) from processed (heat treated) blocks. The ability of *L. monocytogenes* to grow at refrigeration temperatures, while most competing organisms cannot, provides easy survival and proliferation of the organism. Also, this bacterium can survive in biofilms, achieving great persistence in processing environments (Lewis, 2001). Although *L. monocytogenes* can be destroyed if heated to a high enough temperature, there may be post-process contamination of the food product after the heat treatment with subsequent growth. The prevalence of *Listeria* spp. in RTE meats have been variable ranging from 1.8 to 48.0% (Ryu et al., 1992; Wilson, 1995; Bersot et al., 2001; Eleftheriadou et al., 2002; Mena et al., 2004; Vitas et al., 2004; Gibbons et al., 2005). The incidence of *L. monocytogenes* in cold-smoked salmon has been reported to a range between 0% and 75%, with an average prevalence of 10% (Embarek, 1994). Another study reported a prevalence of 7.8% in raw fish, 18.1% in samples from the processing environment, and 7.3% in finished product (Norton et al., 2001a). Additional post-processing hurdles such as antimicrobial packaging are thus needed to inhibit the growth and survival of *L. monocytogenes* in ready-to-eat products like ham steaks and cold-smoked salmon.

Antimicrobial packaging can be a promising tool for protecting RTE meats from *L. monocytogenes* contamination (Janes et al., 2002; Lungu and Johnson, 2005). Antimicrobial packaging films act by preventing microbial growth on a food surface by direct contact of the package with the surface of food. The gradual release

of an antimicrobial substance from a packaging film to the food surface for an extended period of time may be more advantageous than incorporating the antimicrobial into foods. In the latter processes, antimicrobial activity may be lost or reduced due to inactivation of the antimicrobial compound by food components (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

Chitosan is a natural polymer obtained by deacetylation of chitin, which is the major constituent of the exoskeleton of crustaceans. Chitosan has been proved to be nontoxic, biodegradable, and biocompatible. Chitosan has intrinsic antimicrobial activity and inhibits the growth of a wide variety of bacteria (Shahidi et al., 1999; Helander et al., 2001). In an acidic solution, the amine groups on the chitosan molecule are protonated to NH_3^+ and thus acquire a positive charge (Shahidi et al., 1999; Ravi Kumar, 2000). However, neutralized chitosan alone has no effect on bacterial growth when applied to the surface of meat products. Because of its superior film-forming properties, ability to carry antimicrobials, chitosan is a good choice for antimicrobial films. Since edible film formed by chitosan is brittle and does not have good mechanical properties, in this study chitosan was coated onto a plastic film to overcome these shortcomings. The additional benefit of coating chitosan onto plastic films is that chitosan is not consumed with food.

To enhance the efficacy of chitosan-coated film against *L. monocytogenes*, five Generally Recognized as Safe (GRAS) antimicrobials, nisin, sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PB), and sodium benzoate (SB), were

incorporated into the chitosan coating. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, exerts rapid bactericidal effects against gram-positive bacteria, especially against strains of *L. monocytogenes* in laboratory media or model food systems (Delves-Broughton and Gasson, 1994). SL is primarily used as a flavor enhancer in meat and poultry products (Shelef, 1994). SD is a derivative of acetic acid and is used in bread and cakes to prevent mold growth (Jay, 2000). At 0.1 to 0.3%, SD can control growth of *L. monocytogenes* in meat (Schlyter et al., 1993b; Ghanem and Skonberg, 2002). PS is primarily used to control yeasts and molds. Effective antimicrobial concentrations of PS in most foods are in the range of 0.05 to 0.30 % (Sofos and Busta, 1993). El-Shenawy and Marth (1988) found that PS inhibited or inactivated *L. monocytogenes* in a broth substrate, depending on pH and concentration. The antibacterial properties of SB are due to the undissociated form of benzoic acid (Doores, 1993). These studies have investigated the efficacy of these antimicrobials when directly added into or onto food products. An alternative way of controlling *L. monocytogenes* is through their incorporation into a packaging material that is subsequently applied onto food.

The objectives of this project were to (i) evaluate the efficacy of chitosan-coated plastic films alone on controlling the growth of *L. monocytogenes* on ham steaks, (ii) evaluate the potential of chitosan-coated plastic films incorporating antimicrobials to control the growth of *L.monocytogenes* and spoilage organisms on vacuum-packaged ham steaks and cold-smoked salmon, and (iii) investigate the

possible synergistic antimicrobial effects of films incorporating antimicrobials to inhibit growth of *L. monocytogenes* on cold-smoked salmon.

Chapter 2

LITERATURE REVIEW

2.1. *Listeria monocytogenes*

2.1.1. History

Unlike some pathogenic agents responsible for large outbreaks that have marked the history of humans for centuries, the history of *Listeria monocytogenes* is recent. It began officially in 1924. Murray et al. (1926) discovered an organism from a disease that caused sudden death of young rabbits and named it *Bacterium monocytogenes.* In 1927, Pirie (1927) isolated a new microorganism during investigations of unusual deaths observed in gerbils in South Africa, an agent of what he called the"Tiger River disease". He named this new agent *Listerella hepatolytica* and the genus name was dedicated in honor of Lord Lister, "one of the most distinguished of those concerned with bacteriology". In 1940, Pirie proposed the name *Listeria monocytogenes.* The first confirmed isolations of the bacterium from infected individuals, following its initial description, were made in 1929 by Gill from sheep and by Nyfeldt from humans (Gray and Killinger, 1966).The first diagnosis in human was that of a soldier suffering from meningitis at the end of World War I (Cotoni, 1942).

2.1.2. Taxonomy

The listeriae are gram-positive, non-spore forming, and non-acid-fast rods. Six species of *Listeria* are recognized: *L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. ivanovii* and *L. monocytogenes* (Jay, 2000). Only the latter two species are pathogenic to animals. *L. monocytogenes* is the only species causing human illness.

The six species of listeriae are characterized by the possession of antigens that give rise to 17 serovars. Thirteen serotypes of *L. monocytogenes* have been identified, some of which are shared by *L. seeligeri* and *L. innocua*. Only three serotypes (1/2a, 1/2b and 4b) are associated with the majority of sporadic cases of listeriosis; serotype 4b is linked to almost all recent outbreaks (Rocourt and Bille, 1997).

2.1.3. Morphology

Listeria is a facultative intracellular, regular gram-positive rod with rounded ends. Cells are small (0.4-0.5 μm in diameter and 1-2 μm in lengh) and occur singly or in short chains, or they maybe arranged in V or Y forms. In old cultures, some cells lose the ability to retain the Gram stain and may appear to be long, thin and filamentous. *Listeria* does not produce spores and capsules are not formed (Seeliger and Bockemuhl, 1968). The bacterium is motile from its few peritrichous flagella when cultured at 20 to 25 °C (Galsworthy et al., 1990).

Listeria usually grows well on most commonly used bacteriological media. On nutrient agar, colonies are 0.2 to 0.8 mm in diameter, round, smooth, bluish gray,

translucent and slightly raised with fine surface texture and entire margin after 24 h of incubation. During prolonged incubation for 5 to 10 days, well-separated colonies may be 5 mm or more in diameter. The organism forms beta-hemolytic colonies on blood agar plates and blue-green translucent colonies on colorless solid media under obliquely transmitted light, which distinguish *L. monocytogenes* from other *Listeria* species (Farber and Peterkin, 1991).

2.1.4. Growth

The temperature limits of growth of *Listeria* are around 1-45°C with optimum growth temperature at 30-37 °C, but two strains of *L. monocytogenes* have been discovered to even grow at 0.5 °C (Juntilla et al., 1988). The ability to grow at very low temperature was first used by Gray et al. (1948) for selective enrichment of a contaminated sample.

Listeriae grow best in the pH range 6-8 and in general some strains will grow over the pH range of 4.1 to around 9.6. *L. monocytogenes* can grow in 10% (w/v) NaCl and survive at higher concentrations. Survival at low pH and high salt concentration depends strongly on temperature (Cole et al., 1990). It is one of the few foodborne pathogens that can grow at a_w below 0.93.

Listeria is able to grow under aerobic, microaerophilic and anaerobic conditions, is catalase-positive and oxidase-negative. All strains grow on glucose. Only hexoses and pentoses support growth anaerobically; maltose and lactose support growth of some stains aerobically, but sucrose does not (Pine et al., 1989). Lactate,

acetate and acetoin are formed under aerobic conditions as main end products whereas acetoin is not produced under anaerobic conditions (Romick et al., 1996).

2.1.5. Distribution

L. monocytogenes is widely present in nature and can be found in plant, soil, water, silage, sewage, slaughterhouse waste, and human and animal feces. It has been isolated from cattle, sheep, goats, and poultry, but infrequently from wild animals. It is well established that any fresh food product of animal or plant origin may harbor varying numbers of *L. monocytogenes*. In general, the organism has been found in raw milk, soft cheeses, fresh and frozen meat, poultry, and seafood products, and on fruit and vegetable products.

2.2. Human Listeriosis

2.2.1. Hosts at risk

L. monocytogenes has been estimated by the Centers for Disease Control and Prevention (CDC) to cause 2,493 illnesses, 2,322 hospitalizations, and 499 deaths per year in the United States, 99% of which are via consumption of contaminated foods (Mead et al., 1999). Some of the predisposing conditions often associated with listeriosis include neoplastic disease, immunosuppression, pregnancy, extremes of age, diabetes mellitus, alcoholism, cardiovascular and renal collagen diseases, and hemodialysis failure (Nieman and Lorber, 1980). The highest incidence of listeriosis has been in persons over 60 years old and in newborns. Pregnant women are about 20 times more likely than other healthy adults to contact listeriosis. About one-third of

listeriosis cases happen during pregnancy. Newborns rather than the pregnant women themselves suffer the serious effects of infection in pregnancy. The majority of human cases of listeriosis occur in individuals who have an underlying condition which leads to suppression of their T-cell-mediated immunity. In this regard, AIDS patients are almost 300 times more likely to get listeriosis than people with normal immune systems. Healthy adults and children occasionally get infected with *Listeria*, but they rarely become seriously ill.

2.2.2. Infection Dose

The infective dose of *L. monocytogenes* is unknown but is believed to vary with the virulence of the strain and susceptibility and immune status of the victim. From cases contracted through raw or supposedly pasteurized milk, it is safe to assume that in susceptible people, fewer than 1,000 total organisms may cause disease (CFSAN, 2006).

2.2.3. Syndromes

Listeriosis in humans is not characterized by a unique set of symptoms because the course of the disease depends on the state of the host. When susceptible adults contact the disease, meningitis and sepsis are the most common recognized symptoms. Central nervous system infection with *L. monocytogenes* is typically meningitic or encephalitic and usually presents with prodromal symptoms including headache, vomiting, fever, and malaise before the appearance of focal signs of central nervous system infection (Farber and Peterkin, 1991). Pregnant females who contact

the disease may not present any symptoms, but when they do, they are typically mild and influenzalike. Abortion, birthpremature, or stillbirth is often the consequence of listeriosis in pregnant women (Jay, 2000). Two clinical forms of neonatal listeriosis, early- and late-onset forms, are known. The mean incubation time for onset of symptoms for the former disease is 1.5 days and presumably occurs in infants infected in utero. The disease is known as granulomatosis infantisepticum. In late onset neonatal listeriosis, the mean onset of symptoms is 14.3 days, with meningitis as the predominant form of the disease. The mortality rate in systemic listeriosis has been estimated as between 20% and 40% (Farber and Peterkin, 1991). When listeric meningitis occurs, the overall mortality may be as high as 70%; from septicemia 50%, from perinatal/neonatal infections greater than 80%. In infections during pregnancy, the mother usually survives (CFSAN, 2006a). The most effective drugs for treatment are coumermycin, rifampicin and ampicillin, with the last plus an aminoglycoside antibiotic being the best combination (Espaze and Reynaud, 1988).

2.2.4. Food-borne Outbreaks

Most cases of human listeriosis appear to be sporadic although a portion of these sporadic cases may be previously unrecognized common-source clusters (Ciesielski et al., 1988). The source and route of infection are usually unknown. However, the recent association of *L. monocytogenes* with several large foodborne outbreaks suggests that contaminated food may be the primary source of the organism. Between January 1, and June 14, 1985, 86 cases of *L. monocytogenes* infection from

Mexican-style fresh cheeses were identified and 29 deaths occurred in Los Angeles and Orange Counties, California (CDC, 1985). A significant outbreak occurred with frankfurters in 1998 and 1999, which resulted in 21 fatalities and approximately 100 reported cases of listeriosis (CDC, 1999). In 2000, 29 illnesses and 7 deaths caused by *L. monocytogenes* due to consumption of deli turkey meat were identified in 10 states (CDC, 2000). Homemade Mexican-style cheese produced from contaminated raw milk was discovered to be responsible for 12 illnesses of listeriosis in North Carolina in November, 2000 (CDC, 2001). In 2002, another notable outbreak occurred in the northeastern United States, resulting in 10 deaths from the consumption of sliced turkey deli meat (CDC, 2002).

2.2.5. Prevalence of *Listeria monocytogenes* in ready-to-eat foods

Ready-to-eat (RTE) foods including red meats, poultry, seafoods and vegetables have been documented to serve as vehicles for several bacterial pathogens. The pronounced transformation of dietary habits has led to the manufacture of a vast variety of RTE foods. *L. monocytogenes*, because of its ability to survive and multiply in vacuum and gas-packaged products at refrigerated temperature have been recovered from RTE foods worldwide (Duffy et al., 1994; Huss et al., 2000).

2.2.5.1. Prevalence of *Listeria monocytogenes* in ready-to-eat meat products

RTE meat products are the most popular meat products in the United States. Many of these foods involve cooked meat products (cooked ham, bologna, galantine, poultry cold cuts, etc), which are prepared in small portions (slices, steaks,

small pieces, etc.) from processed blocks. The presliced and prepacked cooked ham market is developing at a rapid rate and in many countries has become the main way of product distribution. However, RTE meat products are occasionally contaminated with *L. monocytogenes* mostly due to postprocessing contamination (Tompkin, 2002). Although RTE meat products are fully cooked, either in casings or cook-in bags, they are often reexposed to the processing environment to be packaged in a final retail packaging wrap. The reexposure of product to the processing environment after cooking could lead to possible contamination by bacteria that may be present on conveyors, on metal surfaces, in condensation drippage, in contaminated air filters, in splashed standing water, or on the workers themselves. *L. monocytogenes* can survive in biofilms, achieving great persistence in processing environments. The prevalence of *Listeria* spp. in RTE meats have been variable ranging from 1.8 to 48.0% (Ryu et al., 1992; Wilson, 1995; Bersot et al., 2001; Eleftheriadou et al., 2002; Mena et al., 2004; Vitas et al., 2004; Gibbons et al., 2005). From 1993 to 2000, this pathogen was detected in 4.6 to 8.1% of sliced canned ham and sliced canned luncheon meat which was among the higher levels of incidence among RTE meat products examined by FSIS monitoring and verification program (Farber et al., 2007). Of the random samples collected and analyzed by the FSIS between January 1 and September 30, 2003, 0.75% of RTE meats tested were positive for *L. monocytogenes* (USDA-FSIS, 2003). Due to its ability to grow at refrigerated temperature and its resistance to salt and nitrite (Tompkin, 2002), any *L. monocytogenes* in cured or uncured RTE meat

products, which usually have long shelf-life and are consumed directly without further heating, could proliferate to a threatening level during refrigerated storage. Between January 1994 and October 2006 at least 175 separate Class I or voluntary recalls were issued for RTE meat contaminated with *L. monocytogenes* in the United States, including 74 for deli meats, 42 for sausages, 37 for hot dogs, and 22 for other products (Farber et al., 2007). Because of its high mortality rate and economic impact due to product recalls, *L. monocytogenes* is a major food safety issue for processed meat industry. Currently, the USDA has a "zero tolerance" policy for *L. monocytogenes* in RTE meat products.

2.2.5.2. Prevalence of *Listeria monocytogenes* in cold-smoked salmon

Prevalence of *L. monocytogenes* in smoked fish products is often a concern. Cold-smoking of fish typically involves temperatures in the region of 20–50 °C and does not generate sufficient heat to inactivate this organism that may be present in fish (Eklund et al., 1995; Guyer and Jemmi, 1991). While phenolic and other volatile compounds in smoke may inhibit the growth of *L. monocytogenes* (Eklund et al., 2004; Jemmi and Keusch, 1992).They may not be able to eliminate the bacterium and recontamination could happen during the post-processing stage. Moreover, during storage at refrigeration temperatures growth of *L. monocytogenes* is possible. Previous studies from several countries have reported a variable but occasionally high prevalence of this pathogen in the fish-smoking industry. In a review of cold-smoked salmon samples, Embarek (1994) reported a contamination rate of

between 0 and 75%, with an average prevalence of 10%. Heinitz and Johnson (1998) reported that 17.5% of cold-smoked fish and 8.1% of hot-smoked fish from the United States contained *L. monocytogenes.* An American study reported a prevalence of 7.8% in raw fish, 18.1% in samples from the processing environment, and 7.3% of finished product (Norton et al., 2001a). A Danish study reported contamination of 34–60% of cold-smoked fish with *L. monocytogenes*, and large variability among processing establishments (Jorgensen and Huss, 1998). In studies in Norway (Rørvik and Yndestad, 1991), Switzerland (Jemmi et al., 2002), Canada (Farber, 1991) and New Zealand (Hudson et al., 1992), the pathogen was isolated from 9, 12, 31 and 66% of the cold-smoked salmon samples examined, respectively. Recent studies indicate that raw materials rarely seem to be responsible for finished product contamination in the production of cold-smoked seafood. Instead, the processing environment is the primary source for *L. monocytogenes* contamination (Autio et al., 1999; Norton et al., 2001b; Rørvik et al., 2000; Vogel et al., 2001). Jin et al. (1994) did not detect the bacterium in raw salmon; however, they found 16% of smoked salmon positive for *L. monocytogenes*. *L. monocytogenes* contamination is thus of a great concern for the smoked fish industry.

2.3. Antimicrobials

2.3.1. Chitosan

2.3.1.1. Chemical structure and Properties.

Chitosan is a modified, natural carbohydrate polymer derived by deacetylation of chitin (poly-β-(1 → 4)-N-acetyl-D-glucosamine), a major component of the shells of crustacean such as crab, shrimp, and crawfish and the second most abundant natural biopolymer after cellulose (No and Meyers, 1995). It is composed primarily of glucosamine (2-amino-2-deoxy-ß-D-glucose) and lesser amounts of *N*-acetylglucosamine (Figure 1). Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively (Furusaki et al., 1996). Chemical modifications of these groups have provided numerous useful materials in different fields of application (Kurita, 1986). During the past several decades, chitosan has received increased attention for its commercial applications in the biomedical, food, and chemical industries (Muzzarelli, 1977; Knorr, 1984; Li et al., 1992). Chitosan is now widely produced commercially from crab and shrimp shell wastes with different deacetylation grades and molecular weights (thus, viscosities of chitosan solutions), and, hence, different functional properties (No and Meyers, 1995; Cho et al., 1998). Chitosan is not soluble in water but soluble in weak organic acid solutions. Chitosan derivatives in the form of acetate, ascorbate, lactate, and malate are water-soluble. Water-soluble chitosan can also be produced in the form of oligosaccharide by enzymatic or chemical hydrolysis (Jeon et al., 2001).

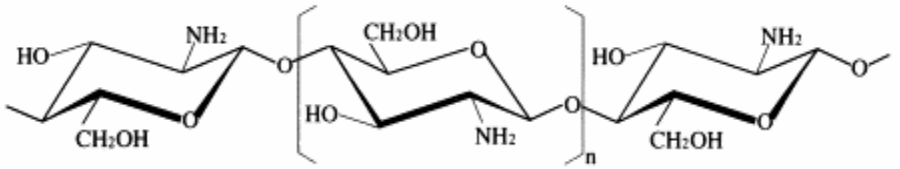

Fig. 2.1 Structure of chitosan.

To date chitosan has attracted notable interest due to its biological activities such as antimicrobial (Sudarshan et al., 1992; No et al., 2002), antitumor (Tokoro et al., 1988), and hypocholesterolemic functions (Sugano et al., 1992). The antimicrobial activity of chitosan against a range of foodborne filamentous fungi, yeast, and bacteria has attracted attention as a potential food preservative of natural origin (Sagoo et al., 2002). In studies on functional properties of chitinous polymers, chitosan has been documented to possess several distinctive properties for use in water and fat uptake, emulsification (Knorr, 1982), dye binding (Knorr, 1983), and gelation (Vorlop and Klein, 1981). Chitosan also possesses a film-forming property allowing its use as edible films or coatings (Butler et al., 1996; Jeon et al., 2002; Nadarajah et al., 2006). Chitosan coating can improve the storability of perishable foods by modifying the internal atmosphere as well as decreasing the transpiration losses (El Ghaouth et al., 1991b; Zhang and Quantick, 1997).

Chitosan is biocompatible, nonantigenic, nontoxic, and biodegradable (Li et al., 1992). Thus, chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively (Weiner, 1992; KFDA, 1995). Biological safety of chitosan has been demonstrated by feeding trials with domestic animals (Hirano et al.,

1990). In 2005, shrimp-derived chitosan was submitted to the US FDA to be considered as GRAS based on the scientific procedures for use in foods in general, including meat and poultry, for multiple technical effects. However, according to GRAS notice number GRN 0001-70, at the notifier's request, the US FDA ceased to evaluate the notice, effective October 31, 2005 (CFSAN, 2006).

2.3.1.2. Antimicrobial activity.

Chitosan has attracted attention as a potential food preservative of natural origin due to its antimicrobial activity against a wide range of foodborne filamentous fungi, yeast, and bacteria (Sagoo et al., 2002). The reported minimimum inhibitory concentrations (MICs) vary widely from 0.0018 to 1.0% and yeasts tend to be more sensitive than bacteria (Knowles and Roller, 2001; Roller and Covill, 2000; Rhoades and Roller, 2000; Sudarshan et al., 1992; Tsai and Su, 1999; Tsai et al., 2000; Wang, 1992). The exact mechanism of the antimicrobial activity of chitosan is still unknown, but several hypotheses have been proposed. The most accepted hypothesis is a change in cell permeability due to interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes. This interaction leads to the leakage of proteinaceous and other intracellular constituents (Young et al., 1982; Papineau et al., 1991; Sudarshan et al., 1992; Fang et al., 1994). Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth (Cuero et al., 1991). It also activates several defense processes in the host tissue (El Ghaouth et al., 1992), acts as a water-binding agent and

inhibits various enzymes (Young et al., 1982). Binding of chitosan with DNA and inhibition of mRNA synthesis occurs via chitosan penetrating the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins (Sudarshan et al., 1992; Hadwiger et al., 1986).

There are several intrinsic and extrinsic factors that affect the antimicrobial activity of chitosan. The antibacterial effects of chitosan and chitosan oligomers are dependent on its molecular weight (Jeon et al., 2001; No et al., 2002), degree of deacetylation (Tsai et al., 2002), and the type of bacterium (No et al., 2002). It has been demonstrated that lower molecular weight chitosans (of less than 10 kDa) have greater antimicrobial activity than native chitosans (Uchida et al., 1989). Highly deacetylated chitosans are more antimicrobial than those with a higher proportion of acetylated amino groups, due to increased solubility and higher charge density. Lower pH increases the antimicrobial activity of chitosan for much the same reasons, in addition to the 'hurdle effect' of inflicting acid stress on the target organisms. Temperature also has an effect, as higher temperature (37°C) has been shown to enhance antimicrobial activity compared to refrigeration temperatures (Tsai and Su, 1999). However, the greatest single influence on antimicrobial activity is the surrounding matrix. Most foods are a mixture of different compounds and many of them may interact with chitosan and lead to loss or enhancement of antibacterial activity. Being cationic, chitosan has the potential to bind to many different food components such as alginates, pectins, proteins and inorganic polyelectrolytes such as

polyphosphate. Solubility can be decreased by using high concentrations of low molecular weight electrolytes such as sodium halides, sodium phosphate and organic anions (Roberts, 1992). These factors mean that, as with most preservative systems, promising results obtained *in vitro* in buffer or microbiological media do not necessarily translate well into real food systems.

2.3.1.3. Applications in foods for improvement of quality and shelf-life.

The use of edible chitosan films and coatings to extend shelf-life and improve the quality of fresh, frozen and fabricated foods has been examined during the past few years due to its biodegradable nature (Kester and Fennema, 1986; Labuza and Breene, 1989). Chitosan has been successfully used as food wraps. The use of *N, O*-carboxymethylchitin films to preserve fruits over long periods has been approved in both Canada and the USA (Davies, 1989).

Due to its ability to form semi-permeable film, chitosan coating can modify the internal atmosphere as well as decrease the transpiration loss (El Ghaouth et al., 1991b) and delay the ripening of fruits (El Ghaouth et al., 1992). Chitosan films are tough, long-lasting, flexible and very difficult to tear. Most of these mechanical properties are comparable to many medium-strength commercial polymers (Butler et al., 1996). Kittur et al. (1998) reported that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values. In contract, Wong et al. (1992) and Butler et al. (1996) observed chitosan films to be good barriers to permeation of oxygen,

while exhibiting relatively low vapor barrier characteristics. Extension of the storage life and better control of decay of peaches, Japanese pears and kiwifruits by application of chitosan film has been documented (Du et al., 1997). Similarly, cucumbers, and bell peppers (El Ghaouth et al., 1991a), strawberries (El Ghaouth et al., 1991b), and tomatoes (El Ghaouth et al., 1992) could be stored for long periods after coating with chitosan. These results may be attributed to decreased respiration rates, inhibition of fungal development and delaying of ripening due to the reduction of ethylene and carbon dioxide evolution. For example, El Ghaouth et al. (1991b, 1992) investigated the effect of chitosan coating on decay and quality of strawberries at 13 °C. Strawberries were inoculated with spore suspensions of *Botrytis cinerea* or *Rhizopus stolonifer* and subsequently dipped in chitosan solutions (1.0 and 1.5% in 0.25 N HCl). In both studies, chitosan coating significantly reduced the decay of strawberries compared to the control. During storage at 4 °C, chitosan-coated berries were firmer, had higher titratable acidity, and synthesized anthocyanin at a slower rate than the control. The improved storability of fresh strawberries by chitosan-based coating also has been documented by Reddy et al. (2000), Han et al. (2005), Park et al. (2005), Hernández-Muñoz et al. (2006), and Vargas et al. (2006).

Effects of chitosan coating on browning of litchi (*Litchi chinensis sonn.*) fruit were investigated. Zhang and Quantick (1997) reported that chitosan coating, irrespective of concentration (1% and 2% dissolved in 2% glutamic acid), delayed changes in contents of anthocyanins, flavonoids, and total phenolics. It also delayed

the increase in polyphenol oxidase (PPO) activity and partially inhibited the increase in peroxidase activity. Jiang et al. (2005) similarly observed that chitosan (2% in 5% acetic acid) coating delayed the decrease in anthocyanin content and the increase in PPO activity. Such effects of chitosan coating were also observed with peeled litchi fruit (Dong et al., 2004), longan fruit (Jiang and Li, 2001), and fresh-cut Chinese water chestnut (Pen and Jiang, 2003).

Several researchers (Lee et al., 1996; Bhale et al., 2003; Caner, 2005) have reported that chitosan coating is effective in preserving the internal quality of eggs without affecting consumer acceptance. Lee et al. (1996) reported that chitosan coating increased the shelf-life of eggs. The Haugh unit value was significantly higher for 2% chitosan (dissolved in 2% acetic acid) coated eggs (45.87) than 1% chitosan (dissolved in 1% acetic acid) coated eggs (24.28) and noncoated eggs (9.38) after 30 d of storage at 20 °C. Caner (2005) also found that the shelf-life of eggs coated with chitosan (3% in 1% acetic acid) could be extended by at least 2 weeks longer compared with that of control at 25 °C, and that overall sensory acceptability for the external quality of chitosan-coated eggs was not different from the control eggs.

Chitosan films containing antimicrobial agents provide a type of active package such that the preservatives released from the film deposit on the food surface and inhibit microbial growth (Labuza and Breene, 1989; Chen et al., 1996). Chen et al. (1996) have observed that the packaging film prepared from methylcellulose, chitosan and preservatives possesses antimicrobial activity.

2.3.2. Nisin

Nisin is a bacteriocin produced by the dairy starter bacterium *Lactococcus lactis* subsp. *lactis*. As first elucidated by Gross and Morrell in 1971, nisin is a pentacyclic peptide composed of 34 amino acids. The molecular weight of nisin is 3,354 daltons, although it usually occurs in dimers or tetramers. Nisin is classified as a lantibiotic. It has a much broader spectrum than most other bacteriocins, being active against a wide range of gram-positive bacteria. In addition, nisin is heat-stable, nontoxic, and sensitive to the action of digestive proteases (Davidson et al., 2005).

Nisin was approved for use in food by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Committee on Food Additives and has achieved GRAS status in the United States (FDA, 1988). The FAO/WHO Committee recommended a maximum daily intake of nisin for a 70-kg person to be 60 mg of pure nisin or 33,000 International units (Hurst and Hoover, 1993). Nisin is mostly used in the food industry to prevent the outgrowth of *Clostridium* spp. in dairy products (especially cheeses) and canned foods. The most established commercially available form of nisin for use as a food preservative is Nisaplin™ with the active ingredient 2.5% nisin A and the predominate ingredients NaCl (77.5%) and nonfat dry milk (12% protein and 6% carbohydrate). Several companies market antimicrobial products containing nisin.

Nisin usually has no effect on gram-negative bacteria, yeasts, and molds, although gram-negative bacteria can be sensitized to nisin by permeabilization of the

outer membrane layer as caused by sublethal heating, freezing, and chelating agents (Delves-Broughton et al., 1996). Normally only gram-positive bacteria are affected. These include lactic acid bacteria, vegetative pathogens such as *Listeria*, *Staphylococcus*, and *Mycobacterium*, and the sporeforming bacteria, *Bacillus* and *Clostridium*. Nisin acts on vegetative cells by inserting into the cytoplasmic membrane, forming pores, and dissipating the proton motive force (Abee, 1995). Damage can range from immediate loss of cellular potassium ions, the hydrolysis and rapid efflux of amino acids and ATP from the cellular membrane, and loss of cellular biosynthesis (Ray, 1992). The spores of bacilli and clostridia are actually more sensitive to nisin than their vegetative cells, although the antagonism is sporostatic, but in these cases, the exact mechanism of action has not been elucidated (Hurst, 1981). Nisin, in conjunction with other inhibitory factors, can provide a barrier for the growth of unwanted contaminating bacteria, thereby reducing the amount of chemicals added to the food, decreasing the intensity of the processing conditions, and contributing to the development of hurdle technologies (Daeschel et al., 1992).

Nisin used alone or combined with other antimicrobials agents or preservation methods have been shown to be valuable in the effort to control *L. monocytogenes* in meats. The activity of nisin alone at concentrations of 400 and 800 IU/g and in combination with 2% sodium chloride against *L. monocytogenes* in minced raw buffalo meat was examined by Pawar et al. (2000). Samples of the raw meat mince were inoculated with 10^3 CFU/g of *L. monocytogenes* and stored at 4 °C.

The counts of *L. monocytogenes* in the control samples increased from 3.0 to 6.4 log CFU/g after 16-d storage; however, nisin significantly inhibited the growth of *L. monocytogenes*. Addition of nisin at a level of 400 IU/g increased the lag phase of *L. monocytogenes*, and at a level of 800 IU/g resulted in counts of *L. monocytogenes* 2.4 log lower than the control samples after 16-d storage. When the storage temperature was increased to 37 °C, the inhibition effects of nisin were less pronounced. Addition of 2% sodium chloride in combination with nisin was found to increase the efficacy of nisin at both storage temperatures.

Murray and Richard (1997) evaluated the antilisterial activity of nisin A and pediocin AcH in the decontamination of artificially contaminated pieces of raw pork. Nisin A was considerably more efficient than pediocin AcH, but after 2 d of storage, surviving bacteria in meat treated with each bacteriocin resumed growth at a rate similar to that of the control. Moreover, nisin was found to be more stable than pediocin AcH.

Samelis et al. (2001) evaluated dipping solutions of nisin with or without organic acids or salts, as inhibitors of *L. monocytogenes* introduced on sliced cooked pork bologna before vacuum packaging and storage at 4°C for 120 days. Inoculated slices were immersed in nisin (5000 IU/ml), or in lactic or acetic acid, sodium acetate or diacetate, and potassium benzoate or sorbate, each combined with nisin. Nisin reduced *L. monocytogenes* by 1.0–1.5 log CFU /cm^2 at treatment followed by a listeriostatic effect for 10 days. Based on the antilisterial efficacy and effects of

treatments on product pH, nisin with 3% SD may be the most promising combination in dipping solutions to control *L. monocytogenes* on sliced cured pork bologna.

The effectiveness of nisin to control growth of *L. monocytogenes* in cold-smoked fish has also been demonstrated by several studies. Nilsson et al. (1997) investigated the antilisterial effect of nisin in combination with carbon dioxide and low temperature in cold-smoked salmon. Addition of nisin (500 or 1000 IU/g) to salmon inoculated with *L. monocytogenes* and stored at 5 °C delayed, but did not prevent growth of *L. monocytogenes* in vacuum-packs. The antilisterial effect of nisin was improved in the presence of 100% CO_2 and increasing NaCl concentrations. Addition of nisin to CO_2 packed cold-smoked salmon resulted in a 1 to 2 log reduction of *L. monocytogenes* followed by a lag phase of 8 and 20 days in salmon with 500 and 1000 IU nisin/g, respectively. The levels of *L. monocytogenes* remained below 10^3 CFU/g during 27 d of storage at both concentrations of nisin.

In a study using vacuum-packed cold-smoked rainbow trout, Nykänen et al. (2000) examined the inhibition of *L. monocytogenes* and mesophilic aerobic bacteria by nisin, SL, or their combination. Trout samples were stored at 8°C for 17 d or at 3°C for 29 d. Both nisin and SL inhibited the growth of *L. monocytogenes* in smoked fish, but the combination of the two compounds was even more effective. The combination of nisin and SL injected into smoked fish decreased the count of *L. monocytogenes* from 3.3 to 1.8 log CFU/g over 16 d of storage at 8 °C. The level of

L. monocytogenes remained almost constant (4.7 to 4.9 log CFU/g) for 29 d at 3°C in the samples injected before smoking which contained both nisin and SL.

2.3.3. Sodium lactate (SL)

Lactic acid is one of the most widely distributed acids in nature, and together with acetic acid is the most widely used preservative. SL, the sodium salt of lactic acid, is GRAS and approved as direct ingredients for use in foods. SL ($CH_3CHOHCOONa$, mol wt 112.07) is generally available as 60% aqueous solution with a neutral pH. It is used as a humectant and flavor enhancer in meat and poultry products at up to 4.8% (by weight of the total formulation), and as a means of inhibiting certain pathogenic bacteria (Code of Federal Regulations, 2000). This antimicrobial prolongs shelf-life by lowering the water activity of foods (Debevere, 1989). The ability of SL to lower water activity is not its only mechanism of inhibition. Another possible mechanism of inhibition has been attributed to the entry of the undissociated form of the weak lipophilic acids into microbial cells and dissociation within cells and acidification of the cell interior. The cell reacts by removing protons to maintain an internal pH of near neutral, and much of the cell's energy is expended and the growth rate is reduced (Shelef, 1994). SL has shown bacteriostatic activity against many types of microorgnisms (Stillmunkes et al., 1993) especially *L. monocytogenes, C. botulinum* and lactic acid bacteria. Some researchers reported that SL increased lag phase, and decreased the growth rate of *Staphylococcus aureus* and *Salmonella* Typhimirium (DeWit and Rombouts, 1990).

Since 1989, the potential benefits of SL as an antimicrobial agent spurred interest in research on its effects in meat products. Anders et al. (1987) reported that *C. botulinum* in fish, chicken and poultry meats was inhibited by the addition of SL at the levels of 1.5–3.5%. The production of toxin by *C. botulinum* in turkey meats was delayed by the addition of SL as well (Maas et al., 1989). Shelef and Yang (1991) found that SL (4%) suppressed *L. monocytogenes* growth in sterile comminuted chicken or beef when incubated at 5, 20, and 35°C. They concluded that lactate contributed to water-holding capacity and increased cooking yields. Inhibition of *L. monocytogenes* by SL was found to increase with increasing fat content and decreasing temperature (Hu and Shelef, 1996). SL holds great potential in preventing the growth of *L. monocytogenes* in RTE food products that are held at refrigeration temperature. In complimentary studies, effective inhibition of *L. monocytogenes* was obtained for up to 4 weeks by incorporating 2% SL in vacuum-packed meat products stored at temperatures from 1–7°C (Unda et al., 1991; Qvist et al., 1994).

Lactates have been combined with other compounds in multiple-barrier studies. Vacumm-packaged beef was treated with 2% SL, pediocin (1400 IU/g), nisin (1400 IU/g), or Microgard^{TM} (2%). Of all the treatments, SL was the most effective during an 8-week storage period at 3 °C (Rozbeh et al., 1993). Cooked-in-bag ham was formulated with additions of 0.1 or 0.2% SD, 2.5 or 3.3% SL, or 1% buffered citrate (Stekelenburg and Kant-Muermans, 2001). *L. monocytogenes* was inoculated into the cooked ham products at an initial level of 10^4 and 10^2 CFU/g of product,

vacuum-packaged and stored for up to 40 days at 4°C. *L. monocytogenes* was inhibited by SL and 0.2% SD. However, the addition of SD was found to affect the sensory qualities of the product precluding its use. Zhu et al. (2005) reported that 2% SL and 0.1% SD in combination with low-dose irradiation were effective in ensuring the safety of RTE meat products against *L. monocytogenes*. SL increased the firmness and saltiness of turkey hams, but its overall impact on quality was minimal.

Only a limited amount of research has been done involving the inhibition of *L. monocytogenes* in cold-smoked salmon. Comminuted salmon containing was mixed with SL, sodium chloride, and sodium nitrite, inoculated with *L. monocytogenes*, vacuum-packaged and stored at 5 or 10°C. SL displayed synergistic antilisterial activity with nitrite and increasing concentrations of sodium chloride. At 5°C, total inhibition of *L. monocytogenes* was achieved for up to 50 d by 2% SL in combination with 3% sodium chloride. At 10°C, total inhibition was achieved for up to 35 d by 3% SL in combination with 3% sodium chloride, or by 2% SL with 125 ppm sodium nitrite and 3% sodium chloride (Pelroy et al., 1994).

2.3.4. Sodium diacetate (SD)

SD, which contains acetic acid (40%) and sodium acetate, is approved as a GRAS substance for miscellaneous and general-purpose usage (FDA, 2000). The acceptable daily intake of SD for humans is 0 to 15 mg/kg body weight. It is used in cheese spread, malt syrups, butter and wrapping material (Davidson et al., 2005). This antimicrobial is approved as a flavoring agent in meat and poultry products at a level

up to 0.25% by weight of the total formulation (Code of Federal Regulations, 2000). At 0.1 to 0.3%, SD can control growth of *L. monocytogenes* in meat (Glass et al., 2002; Schlyter et al., 1993b). It is used in baked goods because of its inhibitory activity against bread mold and rope-forming bacteria, such as *Bacillus mesentericus* (*subtilis*). In bread and cake products, use levels range from 0.25 to 0.4% (Glabe and Maryanski, 1981).

The minimum inhibitory concentration (MIC) of SD was determined for *L. monocytogenes* by Shelef and Addala (1994). This antimicrobial, at concentrations of 35, 32 and 28 mM, were shown to completely inhibit the growth of *L. monocytogenes* in BHI broth at temperatures of 35, 20 and 5°C respectively. Moye and Chambers (1991) studied the effect of surface application of 1 to 3 mg/cm^2 SD powder to chickens and showed that the shelf-life was extended by about 4 days when held at 2°C. The addition of 0.5% SD to turkey slurries also exhibited a listericidal effect (Schlyter et al., 1993b).

Recently, synergistic effects on inhibition of the growth of *L. monocytogenes* were observed with a combination of SL and SD in frankfurters (Samelis et al., 2002), turkey products (Schlyter et al., 1993b), wieners (Glass et al., 2002), and beef bologna (Mbandi and Shelef, 2002). Inhibition of *L. monocytogenes* Scott A was also observed in brain heart infusion broth at 4 and 10°C with a combination of 1.85% potassium lactate and 0.13% SD (Yoon et al., 2003). Blom et al. (1997) found that a mixture of 2.5% SL and 0.25% acetate prevented the growth of

L. monocytogenes in servelat sausage for 4-6 weeks when stored at 4°C while maintaining the sensory acceptability of the sausage. SD at concentrations of 0.1% to 0.3% and SL at concentrations of 2% to 3% were equally effective as antilisterial compounds in meat with little effect on pH and sensory characteristics (Mbandi and Shelef, 2001). Most processors of RTE meat and poultry products in the United States include sodium or potassium lactate at levels of up to 2% combined with 0.05 to 0.15% SD in the formulation of their products (Tompkin, 2002).

Uhart et al. (2004) tested the effectiveness of combinations of antimicrobials to control *L. monocytogenes* on vacuum-packaged beef franks. Commercial beef franks were dipped in pediocin (6,000 AU), a combination of 3% SD and 6% SL, and a combination of the three antimicrobials. Samples treated with the SD–SL combination showed about a 1 log reduction after 2 weeks of storage and between 2 log reductions after 3 weeks of storage. When the three antimicrobials were combined, 1.5 log and 2.5 log reductions were achieved after 2 and 3 weeks of storage, respectively. These results indicate that the use of combined antimicrobial solutions is more effective at inhibiting *L. monocytogenes* than treatments using antimicrobials such as pediocin separately.

The growth of *L. monocytogenes* was also reduced by 2.6 log in crabmeat stored at 4°C for 6 days when treated with 2M SD. Although 1 M SL solution reduced populations within 2 days, populations increased approximately by 0.5 logs within 6 days. Application of 4 M sodium acetate reduced the levels of *L. monocytogenes* by

0.8 log (Degnan et al., 1994). Yoon et al. (2004) evaluated the antimicrobial effects of different levels of PL plus SD mixture against the growth and survival of *L. monocytogenes* Scott A inoculated onto smoked salmon stored at 4 and 10°C. The use of PL in combination with SD at all tested levels (1.5, 3.3, and 5%) completely inhibited the growth of *L. monocytogenes* Scott A on smoked salmon stored at 4°C during 32 days of storage. It also delayed the growth of *L. monocytogenes* Scott A on smoked salmon stored at 10°C for up to 11 days, but a listeriostatic effect was observed only with 5% at 10°C after 11 days. However, the effect of freezing stress was more significant at 4°C than at 10°C, indicating the importance of temperature control of smoked salmon during the retail storage period.

2.3.5. Potassium sorbate (PS)

PS ($CH_3CH=CHCH=CH=COOK$) is the most commonly used salt of sorbic acid. The molecular weight of PS is 150.22, and it constitutes the most soluble form of sorbate. In food systems the solubility of the compound is estimated higher than 50% (Davidson et al., 2005). PS is also a GRAS preservative for miscellaneous and general-purpose usage with a legal limit of 0.3% (Code of Federal Regulations, 2000) when used in accordance with good manufacturing practices. Its primary inhibitory action is against yeasts and molds; its activity against bacteria is not as comprehensive and appears to be selective (Sofos and Busta, 1993). The mechanisms of antimicrobial activity of sorbates are not fully defined. Inhibition of microbial metabolic function is probably the result of morphologic alternations in the structure

of cell, changes in the genetic material, alternations in cell membranes, and inhibition of enzymes or transport functions (Sofos, 1989). Effective antimicrobial concentrations of sorbates in most foods are in the range of 0.05 to 0.30% (Sofos and Busta, 1993). Huhtanen and Feinberg (1980) found that sorbates in meat products (0.30%) had no major adverse effects on sensory qualities such as color and flavor.

Depending on pH and concentration, El-Shenawy and Marth (1988) determined that 0.2% sorbate inhibited or inactivated *L. monocytogenes* in a broth substrate and in a cold-pack cheese food (Ryser and Marth, 1988). PS sensitized cells of *L. monocytogenes* to inactivation by high hydrostatic pressure (Mackey et al., 1995; Palou et al., 1997). In other studies, although sorbate did not affect growth of *L. monocytogenes* in broth, it suppressed cysteine activation of listeriolysin. It was concluded that combinations of sorbate with propionate or lactate, which inhibited growth, could extend shelf-life and increase safety (Kouassi and Shelef, 1995). At the relatively high level of 1%, PS slightly decreased *L. monocytogenes* presence in two commercial cheese brines and thus could be used as an antilisterial agent in commercial brines, but the cost effectiveness of adding high levels (1%) of the substance is questionable, and their long-term stability in the high salt and low pH environment of brines is not well documented (Larson et al., 1999). Additional research using PS alone or in combination with other antimicrobial agents to control the growth of *L. monocytogenes* in cold smoked salmon is needed.

2.3.6. Sodium benzoate (SB)

SB (C_6H_5COONa) was the first chemical preservative approved for use in foods by the U.S. Food and Drug Administration (FDA) (Jay, 2000). It is a white granular or crystalline powder with a molecular weight of 144.1. It is much more soluble than benzoic acid and for this reason it is preferred for use in many cases. The ingredient is used as an antimicrobial agent, flavoring agent or adjuvant. SB is a GRAS preservative up to a maximum permitted level of 0.1% (Code of Federal Regulations, 2007). As a food preservative, its main advantages include low price, ease of incorporation into products and lack of color. Only those organic acids that are lipophilic, such as benzoic acid, have antimicrobial activity that involves interference with the permeability of the microbial cell membrane (Doors, 1993). The antimicrobial activity of SB is related to pH. Like many other food antimicrobials, SB (pKa= 4.20) is most effective in its undissociated form; 60% of the compound is undissociated at pH 4.0. SB is most suitable for foods and beverages with a natural pH below 4.5 or that can be brought into this range by acidification. Currently, SB is used as an antimycotic agent, and most yeasts and fungi are inhibited by 0.05 to 0.1% of the undissociated acid. Food-poisoning bacteria and spore-forming bacteria are generally inhibited by 0.01 to 0.02% undissociated acid.

In a series of studies involving *L. monocytogenes* (El-Shenawy and Marth, 1988; Yousef et al., 1989), it was found that benzoic acid at concentrations of approximately 1000 to 3000 ppm had strong bacteriostatic, but relatively modest

bactericidal, activities against cells in a liquid minimal medium. Incubation of cells in minimal media caused injury that depended on the temperature of incubation but not on the presence of benzoic acid. The concentration of SB required to inhibit growth of *L. monocytogenes* at 4 and 13°C in tryptose broth was 0.05 – 0.1% (500-1000 ppm) at pH 5.0. At pH 5.6, the microorganism was not capable of growth at 4°C with 0.05% SB, while 0.2% was required to inhibit the microorganism (EL-Shenawy and Marth, 1988).

Benzoates are approved in various countries for use as dipping solutions to prevent fungal growth in dry sausages (Sofos, 1989). A study was conducted to evaluate aqueous dipping solutions of organic acids or salts to control *L. monocytogenes* on sliced, vacuum-packaged bologna stored at 4°C for up to 120 days. No significant increase in *L. monocytogenes* populations occurred on bologna slices treated with 2.5 or 5% acetic acid, 5% SD, or 5% potassium benzoate from day 0 to 120 (Samelis et al., 2001). Islam et al. (2002) compared the ability of GRAS chemicals applied to the surfaces of turkey frankfurters to reduce populations of or inhibit the growth of *Listeria monocytogenes.* After 14 days of storage, *L. monocytogenes* counts for frankfurters treated with 25% SB were 3 and 3.5 log less than untreated ones at 4 °C and 13°C, respectively. Frankfurters treated with 25% SB were significantly inhibitory to *L. monocytogenes* when held at 22°C for 7 days or longer. Chicken skin was inoculated with *L. monocytogenes* and wash solutions were evaluated for effectiveness in decontaminating the skin (Hwang and Beuchat, 1995). Washing the

skin with solutions of either 0.3 or 0.5% lactic acid combined with 0.05% SB reduced the numbers of *L. monocytogenes* compared to washing with water. No viable cells from the pathogen were detected on the skin washed with lactic acid/benzoate solutions and stored for 8 days at 4°C.

2.4. Antimicrobial Packaging

Antimicrobial packaging has been touted as a major focus in the next generation of 'active' packaging which has been defined as "a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food". The elaboration of antimicrobial packaging material is a very promising research area. Since microbial contamination of foods occurs primarily at the surface, due to post-processing handling, attempts have been made to improve safety and to delay spoilage. Incorporation of bactericidal agents or growth inhibitors into food formulations or onto food surface by spraying, dipping, or coating without matrix may result in partial inactivation of the active substances and in rapid diffusion within the bulk of food respectively (Quintavalla and Vicini, 2002). The use of packaging film could prove more efficient, by maintaining high concentrations on a food surface with a low migration of active substance. This allows the antimicrobials to be released from the package during a longer period, thus conferring extended activity during the transport and storage phase of the food distribution chain (Torres et al., 1985; Quintavalla and

Vicini, 2002). Moreover, the development of new biodegradable or edible packaging material films has recently been undertaken for environmental reasons.

According to Cooksey (2001), there are three basic categories of antimicrobial films. One involves the direct incorporation of the antimicrobial additive into the packaging film, whereas the second type is coated with a material that acts as a carrier for the additive. These categories of materials can release the antimicrobial agents onto the surface of the food (Appendini and Hotchkiss, 2002). Third, antimicrobial macromolecules with film-forming properties, such as cationic amino-polysaccharides that can produce antimicrobial films (Ouattara et al., 2000; Coma et al., 2002; Bégin and Van Calsteren, 1999). Release of a biocide agent would not be required by this system. Moreover, legal issues and standards concerning the rate of migration of substances in packaging into food products do not limit the development of such bioactive materials. However, the limitation is the necessity of direct contact between the packaging and the food for these systems to be effective.

2.4.1. Antimicrobial films incorporating GRAS preservatives

Chitosan forms a strong film that can carry high levels of antimicrobials. Chitosan is a good choice for antimicrobial films because of its superior film-forming properties, ability to adsorb nutrients used by bacteria (Darmadji and Izumimoto, 1994), and capacity to bind water and inhibit various bacterial enzyme systems (Young et al., 1982). However, neutralized chitosan alone has no effect on bacterial growth when applied to the surface of meat products.

Vojdani and Torres (1990) chose polysaccharide films such as chitosan, methylcellulose (MC) and hydroxypropylmethylcellulose (HPMC) as a carrier for PS. They indicated that sorbate eventually absorbs into the food from the surface and therefore, the protective effect is lost. They hypothesized that the film coatings could help retain the levels of sorbate at the surface of the food depending on how much PS diffused through the film. They determined that all polysaccharide films with sorbate could improve surface protection of foods with an estimated effective durability of 1.5-2.0 months at room temperature and 3.5-5 months at refrigeration temperatures. Chitosan was found to have the highest permeation while MC and HPMC combination had the lowest. Films with the lowest were considered the most desirable because it allowed sorbate to diffuse to the surface of the food over a longer period of time.

Coma et al. (2002) evaluated the antilisteria effects of edible chitosan coatings showed by numeration and epifluorescence methods that thus impart a strong localized functional effect at the food surface. The use of film-forming solution in culture liquid medium showed the known flocculant phenomenon combined with bactericidal activity, but preserving 20% of the initial microbial load as viable cells in the flocs. However, chitosan film showed 100% inhibition of *L. monocytogenes* for at least 8 d, as demonstrated by bactericidal activity measurements by epifluorescence assays. A decrease in antibactericidal effect with time was observed, most probably due to a decreasing availability of amino groups of chitosan. Later results were

validated on Emmental cheese samples using *L. innocua* as a surrogate strain because of its nonpathogenicity.

Moller et al. (2004) evaluated the antimicrobial efficiency of chitosan/HPMC films, chitosan/HPMC films associated with lipid, and chitosan/HPMC films chemically modified by cross-linking. In addition, the physicochemical properties of composite films were evaluated to determine their potential for food applications. Anti-listerial activity of films was determined on solid medium by a numeration technique. Chitosan/HPMC-based films, with and without stearic acid, inhibited the growth of *L. monocytogenes* completely.

Antimicrobial and physicochemical properties of chitosan films and chitosan films enriched with essential oils (EO) were determined by Zivanovic et al. (2005) in vitro and on processed meat. Antimicrobial effects of pure EO, and of chitosan-essential oil films against *L. monocytogenes* and *Escherichia coli* O157:H7 were determined by an agar diffusion test. The antibacterial effects of the EO were similar when applied alone or incorporated in the films. Pure chitosan films reduced *L. monocytogenes* by 2 log, whereas the films with 1 and 2% oregano EO decreased the numbers of *L. monocytogenes* by 3.6 to 4 log and *E. coli* by 3 log. During application on bologna discs, the films absorbed moisture. The films have the potential to be used as active biodegradable films with strong antimicrobial effects.

Pranoto et al. (2005) compared antimicrobial effect of chitosan edible film incorporating garlic oil (GO) with conventional food preservative PS and bacteriocin

nisin at various concentrations. Incorporation of GO up to levels at least 100 µl/g, PS at 100 mg/g or nisin at 51,000 IU/g of chitosan were found to have antimicrobial activity against *L. monocytogenes*. At these levels, the films were physically acceptable in terms of appearance and mechanical and physical properties. GO components did not affect the physical and mechanical properties of chitosan films as they did not interact with the functional groups of chitosan as measured by Fourier Transform Infrared.

Nisin packaging is a promising form of active food packaging for ready-to-eat foods. Ko et al. (2001b) studied the physical and chemical properties of soy protein isolates, wheat-gluten- and egg-albumin-based films containing nisin and the resulting antimicrobial action against *L. monocytogenes*. Films with higher hydrophobicity values (280 to 450 units) under an acidic environment exerted a greater inhibitory effect against *L. monocytogenes*. Grower et al. (2005) found that low-density polyethylene (LDPE) film could be coated with a solution containing either a high or low molecular weight MC or HPMC incorporating nisin. Films prepared contained 10,000, 7500, 5000, 2500, or 0 IU/cm^2 nisin. The coated-film samples were found to be effective for inhibition of *L. monocytogenes*, particularly when 7500 and 10,000 IU/cm^2 nisin were used.

Li et al. (2006) studied effect of konjac glucomannan (KGM) edible fllm incorporating chitosan and nisin at various ratios or concentrations against pathogenic bacteria. Incorporation of nisin at 463 IU per disk of film for the selected KC2 (mixing

ratio KGM 80/Chitosan 20) produced antimicrobial activity against *S. aureus*, *L. monocytogenes*, and *B. cereus*. The mean values of inhibition zone diameters of the CHI-Nisin series and the KC2-Nisin series were higher than those of the KGM-Nisin series at each corresponding concentration and they were significantly different ($P < 0.05$); however, there was no significant difference in the antimicrobial effect between chitosan and KC2 incorporating nisin. At all these levels, the blend film KC2-nisin had a satisfactory appearance, mechanical and physical properties, and antimicrobial activity.

Other traditional preservatives such as SB, SD and SL have also been incorporated into antimicrobial films. Vartiainen et al. (2003) incorporated traditional food preservatives such as PS, SL, SD and SB into synthetic plastics, LDPE, polymaleic acid-co-olefine, polystyrene and polyethylene terephthalate (PET), aimed at producing antimicrobial packaging material for foodstuffs. The films were tested for antimicrobial activity against *Bacillus subtilis*, *E. coli* and *Aspergillus niger*. Antimicrobial substances added into polystyrene and PET produced the strongest activities; however, due to the brittle structure of these materials, they were not tested further.

Limjaroen et al. (2003) also investigated the feasibility of incorporating chemical preservatives or antimicrobial agents into a plastic film to control target microorganisms. Antimicrobial coatings were developed by incorporation of nisin as well as traditional preservatives such as SD, sorbic acid, and PS into a coating material.

Films containing nisin, sorbic acid, and PS inhibited *L. monocytogenes* strain CWD 95. The lowest level of nisin, sorbic acid, and PS that had antimicrobial activity was 1, 1.5, and 2% (w/v) respectively. In addition, films incorporating sorbic acid were the most homogeneous.

Kristo et al. (2008) prepared antimicrobial films by incorporating different concentrations of SL, PS and nisin into sorbitol-plasticized sodium caseinate (SC) films. Their antimicrobial effectiveness against *L. monocytogenes* was studied as a function of antimicrobial concentration. Nisin-containing SC films were the most effective in reducing growth of *L. monocytogenes*, followed by PS-impregnated SC films, whereas films containing SL were slightly effective in this respect and only at the higher concentration (40% w/w film dry basis). The results indicated that for effective applications of antimicrobial coatings in foods, sufficient knowledge is required not only on the independent properties of the coating film and the antimicrobial compound but also on their interactions.

References

Abee, T. (1995) Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. *FEMS Microbiol. Lett.* 129, 1-9.

Anders, P. J., Milkowski, A. L. and Cereveny, J. G. (1987) A food stuff containing a lactate salt.

Appendini, P. and Hotchkiss, J. H. (2002) Review of antimicrobial food packaging. *Innov. Food Sci. Emerg. Technol.* 3, 113-126.

Autio, T., Hielm, S., Miettinen, M., Sjoberg, A. M., Aarnisalo, K. and Bjorkroth, J. (1999) Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Appl. Environ. Microbiol.* 65, 150-155.

Bégin, A. and Van Calsteren, M. (1999) Antimicrobial films produced from chitosan. *Int. J. Biol. Macromol.* 26, 63-67.

Bersot, L. S., Landgraf, M., Franco, B. D. G. M. and Destro, M. T. (2001) Production of mortadella: Behavior of *Listeria monocytogenes* during processing and storage conditions. *Meat Sci.* 57, 13-17.

Bhale, S., No, H. K., Prinyawiwatkul, W., Farr, A. J., Nadarajah, K. and Meyers, S. P. (2003) Chitosan coating improves shelf life of eggs. *J. Food Sci.* 68, 2378-2383.

Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E. and Nissen, H. (1997) Addition of 2.5% lactate and 0.25% acetate controls growth of *Listeria monocytogenes* in vacuum-packed, sensory-acceptable servelat sausage and cooked ham stored at 4 degrees C. *Int. J. Food Microbiol.* 38, 71-76.

Butler, B. L., Vergano, P. J., Testin, R. F., Bunn, J. M. and Wiles, J. L. (1996) Mechanical and barrier properties of edible chitosan films as affected by composition and storage. *J. Food Sci.* 61, 953.

Caner, C. (2005) The effect of edible eggshell coatings on egg quality and consumer perception. *J. Sci. Food Agric.* 85, 1897-1902.

CDC. (1985) Epidemiologic notes and reports of listeriosis outbreak associated with mexican-style cheese-california. *Morb. Mort. Wkly. Rep.* 34, 357-359.

CDC. (1999) Update: Multistate outbreak of listeriosis—United States, 1998–1999. *Morb. Mort. Wkly. Rep.* 47, 1117-1118.

CDC. (2000) Multistate outbreak of listeriosis—United States, 2000. *Morb. Mort. Wkly. Rep.* 49, 1129-1130.

CDC. (2001) Outbreak of listeriosis associated with homemade Mexican-style cheese-North Carolina, October 2000-January 2001. *Morb. Mort. Wkly. Rep.* 50, 560-562.

CDC. (2002) Public health dispatch: Outbreak of listeriosis—Northeastern United States, 2002. *Morb. Mort. Wkly. Rep.* 51, 950-951.

Center for Food Safety and Applied Nutrition (CFSAN). (2006a) Foodborne pathogenic microorganisms and natural toxins handbook.

CFSAN. (2006b) Inventory of GRAS notices: Summary of all GRAS notices. http://www.cfsah.fda.gov/~rdb/opa-gras.html.

Chen, M., Yeh, G. H. and Chiang, B. (1996) Antimicrobial and physicochemical properties of methylcellulose and chitosan films containing a preservative. *J. Food Process. Preserv.* 20, 379-390.

Cho, Y. I., No, H. K. and Meyers, S. P. (1998) Physicochemical characteristics and functional properties of various commercial chitin and chitosan products. *J. Agric. Food Chem.* 46, 3839-3843.

Ciesielski, C. A., Hightower, A. W., Parsons, S. K. and Broome, C. V. (1988) Listeriosis in the United States: 1980-1982. *Arch. Intern. Med.* 148, 1416-1419.

Code of Federal Regulations, U.S. FDA, 2000. Available at http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/99-028DF.htm

Code of Federal Regulations, U.S. FDA, 2007. Available at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=184.1733

Cole, M. B., Jones, M. V. and Holyoak, C. (1990) The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes. J. Appl. Bacteriol.* 69, 63-72.

Coma, V., Martial-Gros, A., Garreau, S., Copinet, A., Salin, F. and Deschamps, A. (2002) Edible antimicrobial films based on chitosan matrix. *J. Food Sci.* 67, 1162-1169.

Cooksey, K. (2001) Antimicrobial food packaging materials. *Addit. Polym.* 8, 6-10.

Cotoni, L. (1942) A propos des bacteries denommees *Listerella*-rappel d'une observation ancienne de meningite chez l'homme. *Ann. Inst. Pasteur.* 68, 95.

Cuero, R. G., Osuji, G. and Washington, A. (1991) *N*-carboxymethyl chitosan inhibition of aflatoxin production: Role of zinc. *Biotech. Lett.* 13, 441-444.

Daeschel, M. A., McGuire, J. and Al-Makhlafi, H. (1992) Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic silicon surfaces. *J. Food Prot.* 55, 731-735.

Darmadji, P. and Izumimoto, M. (1994) Effect of chitosan in meat preservation. *Meat Sci.* 38, 243-254.

Davidson, P. M., Sofos, J. N. and Branen, A. L. (2005) *Antimicrobials in food.* Boca Raton, FL: CRC Press.

Davies, D. H., Elson, C. M. and Hayes, E.R. (1989) *N,O*-carboxymethylchitosan, a new water soluble chitin derivatives. In Chitin and chitosan (Eds Skjak-bræk, G., Anthonsen, T. and Sandford, P.). pp. 467-472. London, U.K. Elsevier.

Debevere, J. M. (1989) The effect of sodium lactate on the shelf-life of vacuum-packed coarse liver pate. *Fleischwirtsch.* 69, 223-224.

Degnan, A. J., Kaspar, C. W., Otwell, W. S., Tamplin, M. L. and Luchansky, J. B. (1994) Evaluation of lactic acid bacterium fermentation products and foodgrade chemicals to control *Listeria monocytogenes* in blue crab (*Callinectes sapidus*) meat. *Appl. Environ. Microbiol.* 60, 3198-3203.

Delves-Broughton, J. and Gasson, M. J. (1994) Nisin. In Natural antimicrobial systems in food preservation (Eds Dillon, V.M. and Board, R.G.) pp. 99-132. Wallingford, UK. CAB International.

Delves-Broughton, J., Blackburn, P., Evans, R. J. and Hugenholtz, J. (1996) Applications of the bacteriocin, nisin. *Antonie van Leeuwenhoek.* 69, 193-202.

De Wit, J. C. and Rombouts, F. M. (1990) Antimicrobial activity of sodium lactate. *Food Microbiol.* 7, 113-120.

Dong, H. Q., Cheng, L. Y., Tan, J. H., Zheng, K. W. and Jiang, Y. M. (2004) Effects of chitosan coating on quality and shelf life of peeled litchi fruit. *J. Food Eng.* 64, 355-358.

Doores, S. (1993) Organic acids. In Antimicrobials in foods, 2nd ed. (Eds P. M. Davidson and Branen, A. L.) pp. 95-136. New York. Marcel Dekker.

Du, J., Gemma, H., Iwahori, S. (1997) Effects of chitosan coating on the storage of peach, Japanese pear, and kiwifruit. *J. Japan. Soc. Hort. Sci.* 66, 15-22.

Duffy, L. L., Vanderlinde, P. B. and Grau, F. H. (1994) Growth of *Listeria monocytogenes* on vacuum-packed cooked meats: Effects of pH, aw, nitrite and ascorbate. *Int. J. Food Microbiol.* 23, 377-390.

Eklund, M. W., Peterson, M. E., Poysky, F. T., Paranjpye, R. N. and Pelroy, G. A. (2004) Control of bacterial pathogens during processing of cold-smoked and dried salmon strips. *J. Food Prot.* 67, 347-351.

Eklund, M. W., Poysky, F. T., Paranjpye, R. N., Lashbrook, L. C., Peterson, M. E. and Pelroy, G. A. (1995) Incidence and sources of *Listeria monocytogenes* in cold-smoked fishery products and processing plants. *J. Food Prot.* 58, 502-508.

El Ghaouth, A., Arul, J., Asselin, A. and Benhamou, N. (1992) Antifungal activity of chitosan on post-harvest pathogens: Induction of morphological and cytological alterations an *Rhizopus stolonifer*. *Mycol. Res.* 96, 769-779.

El Ghaouth, A., Arul, J. and Ponnampalam, R. (1991a) Use of chitosan coating to reduce water loss and maintain quality of cucumber and bell pepper fruits. *J. Food Process. Preserv.*15, 359-368.

El Ghaouth, A., Arul, J., Ponnampalam, R.and Boulet, M. (1991b) Chitosan coating effect on storability and quality of fresh strawberries. *J. Food Sci.* 56, 1618.

Eleftheriadou, M., Varnava-Tello, A., Metta-Loizidou, M., Nikolaou, A. S. and Akkelidou, D. (2002) The microbiological profile of foods in the republic of cyprus: 1991-2000. *Food Microbiol.* 19, 463-471.

El-Shenawy, M. A. and Marth, E. H. (1988) Inhibition or inactivation of *Listeria monocytogenes* by sorbic acid. *J. Food Prot.* 51, 842-847.

Embarek, P. K. B. (1994) Presence, detection and growth of *Listeria monocytogenes* in seafoods: A review. *Int. J. Food Micro.* 23, 17-34.

Espaze, E. P. and Reynaud, A. E. (1988). Antibiotic susceptibilities of *Listeria*: In vitro studies. *Infection.* 16, 160-164.

Fang, S. W., Li, C. F. and Shih, D. Y. C. (1994) Antifungal activity of chitosan and its preservative effect on low-sugar candied kumquat. *J. Food Prot.* 57, 136.

Farber, J. M. and Peterkin, P. I. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55, 476-511.

Farber, J. M. (1991). *Listeria monocytogenes* in fish products. *J. Food Prot.* 54, 922.

Farber, J. M., Pagotto, F. and Scherf, C. (2007) Incidence and behavior of *Listeria monocytogenes* in meat products. In *Listeria,* listeriosis, and food safety, 3rd ed. (Eds. Ryser, E. T. and Marth, E. H.). pp. 503-570. Boca Raton, FL: CRC Press.

FDA. (1988) Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. *Fed. Regist.* 53, 11247-11251.

Furusaki, E., Ueno, Y., Sakairi, N., Nishi, N. and Tokura, S. (1996) Facile preparation and inclusion ability of a chitosan derivative bearing carboxymethyl-β-cyclodextrin. *Carbohydr. Polym.* 29, 29-34.

Galsworthy, S. B., S. Girdler and S. F. Koval. (1997) Chemotaxis in *Listeria monocytogenes. Acta Microbiol. Hung.* 37, 81-85.

Ghanem, A. and Skonberg, D. (2002) Effect of preparation method on the capture and release of biologically active molecules in chitosan gel beads. *J. Appl. Polym. Sci.* 84, 405-413.

Gibbons, I., Adesiyun, A., Seepersadsingh, N. and Rahaman, S. (2006) Investigation for possible source(s) of contamination of ready-to-eat meat products with *Listeria* spp. and other pathogens in a meat processing plant in trinidad. *Food Microbiol.* 23, 359-366.

Glabe, E. F. and Maryanski, J. K. (1981) Sodium diacetate: An effective mold inhibitor. *Cereal Foods World.* 26, 285-289.

Glass, K. A., Granberg, D. A., Smith, A. L., McNamara, A. M., Hardin, M. and Mattias, J. (2002) Inhibition of *Listeria monocytogenes* by sodium diacetate and sodium lactate on wieners and cooked bratwurst. *J. Food Prot.* 65, 116-123.

Gray, M. L. and Killinger, A. H. (1966) *Listeria monocytogenes* and listeric infections. *Bacteriol. Rev.* 30, 309-382.

Gray, M. L., Stafseth, H. J., Thorp, F., Sholl, J. L. B. and Riley, J. W. F. (1948) A new technique for isolating *Listerellae* from the bovine brain. *J. Bacteriol.* 55, 471-476.

Gross, E., and Morell, J. L. (1971) The structure of nisin. *J. Am. Chem. Soc.* 93, 4634-4635.

Grower, J. L., Cooksey, K. and Getty, K. (2004) Release of nisin from methylcellulose-hydroxypropyl methylcellulose film formed on low-density polyethylene film. *J. Food Sci.* 69, 107-111.

Guyer, S. and Jemmi, T. (1991) Behavior of *Listeria monocytogenes* during fabrication and storage of experimentally contaminated smoked salmon. *Appl. Environ. Microbiol.* 57, 1523-1527.

Hadwiger, L. A., Kendra, D. F., Fristensky, B.W. and Wagoner, W. (1985). Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. In Chitin in nature and technology (Eds. Muzzarelli, R. A. A., Jeuniaux, C. and Gooday, G.W.). pp. 209-222. New York. Plenum Press.

Han, C., Lederer, C., McDaniel, M. and Zhao, Y. (2005) Sensory evaluation of fresh strawberries (*Fragaria ananassa*) coated with chitosan-based edible coatings. *J. Food Sci.* 70, 172-178.

Heinitz, M. L. and Johnson, J. M. (1998) The incidence of *Listeria* spp., *Salmonella* spp., and *Clostridium botulinum* in smoked fish and shellfish. *J. Food Prot.* 61, 318-323.

Helander, I. M., Nurmiaho-Lassila, E., Ahvenainen, R., Rhoades, J. and Roller, S. (2001) Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Int. J. Food Microbiol.* 71, 235-244.

Hernandez-Munoz, P., Almenar, E., Ocio, M. J. and Gavara, R. (2006) Effect of calcium dips and chitosan coatings on postharvest life of strawberries (*Fragaria x ananassa*). *Postharvest Biol. Technol.* 39, 247-253.

Hirano, S., Itakura, C., Seino, H., Akiyama, Y., Nonaka, I. and Kanbara, N. (1990) Chitosan as an ingredient for domestic animal feeds. *J. Agric. Food Chem.* 38, 1214-1217.

Hu, A. C. and Shelef, L. A. (1996) Influence of fat content and preservatives on the behavior of *Listeria monocytogenes* in beaker sausage. *J. Food Saf.* 16, 175-181.

Hudson, J. A., Mott, S. J., Delacy, K. M. and Edridge, A. L. (1992) Incidence and coincidence of *Listeria* spp., motile aeromonads and *Yersinia enterocolitica* on ready-to-eat fleshfoods. *Int. J. Food Microbiol.* 16, 99-108.

Huhtanen, C. N. and Feinberg, J. (1981) Sorbic acid inhibition of *Clostridium botulinum* in nitrite-free poultry frankfurters. *J. Food Sci.* 45, 453-457.

Hurst, A. (1981) Nisin. *Adv. Appl. Microbiol.* 27, 85-123.

Hurst, A. and Hoover, D. G. (1993) Nisin. In Antimicrobials in foods (Eds Davidson P.M. and Branen, A.L.) pp. 369-407. New York. Marcel Dekker.

Huss, H. H., Jorgensen, L. V. and Vogel, B. F. (2000) Control options for *Listeria monocytogenes* in seafoods. *Int. J. Food Microbiol.* 62, 267-274.

Hwang, C. A. and Beuchat, L. R. (1995) Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin. *J. Food Prot.* 58, 19-23.

Islam, M., Chen, J., Doyle, M. P. and Chinnan, M. (2002) Control of *Listeria monocytogenes* on turkey frankfurters by generally-recognized-as-safe preservatives. *J. Food Prot.* 65, 1411-1416.

Janes, M. E., Kooshesh, S. and 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. *J. Food Sci.* 67, 2754-2757.

Jay, J. M. (2000) Modern food microbiology, 6th ed. Gaithersburg, MD. Aspen Publishers, Inc.

Jemmi, T. and Keusch, A. (1992) Behavior of *Listeria monocytogenes* during processing and storage of experimentally contaminated hot-smoked trout. *Int. J. Food Microbiol.* 15, 339-346.

Jemmi, T., Pak, S. I. and Salman, M. D. (2002) Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland, 1992-2000. *Prev.Vet. Med.* 54, 25-36.

Jeon, Y., Kamil, J. Y. V. A. and Shahidi, F. (2002) Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. *J. Agric. Food Chem.* 50, 5167-5178.

Jeon, Y., Park, P.and Kim, S. (2001) Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydr. Polym.* 44, 71-76.

Jiang, Y. M. and Li, Y. B. (2001) Effects of chitosan coating on postharvest life and quality of longan fruit. *Food Chem.* 73, 139-143.

Jiang, Y., Li, J. and Jiang, W. (2005) Effects of chitosan coating on shelf life of cold-stored litchi fruit at ambient temperature. *LWT - Food Sci. Technol.* 38, 757-761.

Jin, M., Kusunoki, K., Ikejima, N., Arai, T., Irikura, Y., Suzuki, K., Hirata, I., Kokubo, Y. and Maruyama, T. (1994) Incidence of *Listeria monocytogenes* in smoked salmon. *Jpn. J. Food Microbiol.* 11, 107-111.

Jørgensen, L. V. and Huss, H. H. (1998) Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *Int. J. Food Microbiol.* 42, 127-131.

Junttila, J. R., Niemela, S. I. and Hirn, J. (1988) Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. *J. Appl. Bacteriol.* 65, 321-327.

Kester, J. J. and Fennema, O. R. (1986) Edible films and coatings: A review. *Food Technol.* 40, 47-59.

Kittur, F. S., Kumar, K. R. and Tharanathan, R. N. (1998) Functional packaging properties of chitosan films. *Z. Lebensm. Unters Forsch.* 206, 44-47.

Knorr, D. (1982) Functional properties of chitin and chitosan. *J. Food Sci.* 47, 593-595.

Knorr, D. (1983) Dye binding properties of chitin and chitosan. *J. Food Sci.* 48, 36.

Knorr, D. (1984) Use of chitinous polymers in food--a challenge for food research and development. *Food Technol.* 38, 85.

Knowles, J. and Roller, S. (2001) Efficacy of chitosan, carvacrol, and a hydrogen peroxide-based biocide against foodborne microorganisms in suspension and adhered to stainless steel. *J. Food Prot.* 64, 1542-1548.

Ko, S., Janes, M. E., Hettiarachchy, N. S. and Johnson, M. G. (2001a) Development of a food packaging coating material with antimicrobial properties. *J. Plast. Film Sheet.* 19, 95-109.

Ko, S., Janes, M. E., Hettiarachchy, N. S. and Johnson, M. G. (2001b) Physical and chemical properties of edible films containing nisin and their action against *Listeria monocytogenes*. *J. Food Sci.* 66, 1006-1011.

KFDA (Korea Food and Drug Administration). (1995) *Food additives code.*

Kouassi, Y. and Shelef, L. A. (1995) Listeriolysin O secretion by *Listeria monocytogenes* in broth containing salts of organic acids. *J. Food Prot.* 58, 1314-1319.

Kristo, E., Koutsoumanis, K. P.and Biliaderis, C. G. (2008) Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. *Food Hydrocolloids.* 22, 373-386.

Kurita, K. (1986) Chemical modifications of chitin and chitosan. In Chitin in nature and technology (Eds. Muzzarelli, R. A. A., Jeuniaux, C. and Gooday, G.W.). pp. 287-293. New York. Plenum Press.

Labuza, T. P. and Breene, W. M. (1989) Applications of "active packaging" for improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods. *J. Food Process. Preserv.* 13, 1-69.

Larson, A. E., Johnson, E. A. and Nelson, J. H. (1999) Survival of *Listeria monocytogenes* in commercial cheese brines. *J. Dairy Sci.* 82, 1860-1868.

Lee, S. H., No, H. K. and Jeong, Y. H. (1996) Effect of chitosan coating on quality of egg during storage. *J. Korean. Soc. Food. Nutr.* 25, 288-93.

Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* 45, 999–1007.

Li, B., Peng, J., Yie, X. and Xie, B. (2006) Enhancing physical properties and antimicrobial activity of konjac glucomannan edible films by incorporating chitosan and nisin. *J. Food Sci.* 71, 174-178.

Li, Q., Dunn, E. T., Grandmaison, E. W. and Goosen, M. F. A. (1992) Applications and properties of chitosan. *J. Bioactive Compatible Polym.* 7, 370-397.

Lungu, B. and Johnson, M.G. (2005) Fate of *Listeria monocytogenes* inoculated onto the surface of model turkey frankfurter pieces treated with zein coatings containing nisin, sodium diacetate, and sodium lactate at 4°C. *J. Food Prot.* 68, 855-859.

Maas, M. R., Glass, K. A. and Doyle, M. P. (1989) Sodium lactate delays toxin production by *Clostridium botulinum* in cook-in-bag turkey products. *Appl. Environ. Microbiol.* 55, 2226-2229.

Mackey, B. M., Forestiere, K. and Isaacs, N. (1995) Factors affecting the resistance of *Listeria monocytogenes* to high hydrostatic pressure. *Food Biotech.* 9, 1-11.

Mbandi, E. and Shelef, L. A. (2001) Enhanced inhibition of *Listeria monocytogenes* and *Salmonella enteritidis* in meat by combinations of sodium lactate and diacetate. *J. Food Prot.* 64, 640-644.

Mbandi, E.and Shelef, L. A. (2002) Enhanced antimicrobial effects of combination of lactate and diacetate on *Listeria monocytogenes* and *Salmonella* spp. in beef bologna. *Int. J. Food Microbiol.* 76, 191-198.

Mead, P. S., Slutsker, L., Dietz, V., McCraig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. and Tauxe, R. V. (1999) Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5, 607-625.

Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Hogg, T. and Gibbs, P. A. (2004) Incidence of *Listeria monocytogenes* in different food products commercialized in portugal. *Food Microbiol.* 21, 213-216.

Moller, H., Grelier, S., Pardon, P. and Coma, V. (2004) Antimicrobial and physicochemical properties of chitosan-HPMC-based films. *J. Agric. Food Chem.* 52, 6585-6591.

Moye, C. J. and Chambers, A. (1991) Poultry processing: An innovative technology for *Salmonella* control and shelf life extension. *Food Australia.* 43, 246-249.

Murray, E. G. D., Webb, R. A. and Swann, M. B. R. (1926) A disease of rabbits characterized by a large mononuclear leukocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J. Pathol. Bacteriol.* 29, 407-439.

Murray, M. and Richard, J. A. (1997) Comparative study of the antilisterial activity of nisin A and pediocin AcH in fresh ground pork stored aerobically at 5°C. *J. Food Prot.* 60, 1534-1540.

Muzzarelli, R. A. A. (1977) Chitin. Oxford, U.K. Pergamon.

Nadarajah, K., Prinyawiwatkul, W., No, H. K., Sathivel, S. and Xu, Z. (2006) Sorption behavior of crawfish chitosan films as affected by chitosan extraction processes and solvent types. *J. Food Sci.* 71, 33-39.

Nieman, R. E. and Lorber, B. (1980) Listeriosis in adults: A changing pattern. Report of eight cases and review of the literature, 1968-1978. *Rev. Infect. Dis.* 2, 207-227.

Nilsson, L., Henrik Huss, H. and Gram, L. (1997) Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. *Int. J. Food Microbiol.* 38, 217-227.

No, H. K. and Meyers, S. P. (1995) Preparation and characterization of chitin and chitosan--a review. *J. Aquat. Food Prod. Technol.* 4, 27-52.

No, H. K., Park, N. Y. Lee, S. H. and Meyers, S. P. (2002) Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.* 74, 65-72.

Norton, D. M., McCamey, M. A., Gall, K. L., Scarlett, J. M., Boor, K. J. and Wiedmann, M. (2001a) Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl. Environ. Microbiol.* 67, 198-205.

Norton, D. M., Scarlett, J. M., Horton, K., Sue, D., Thimothe, J. and Boor, K. J. (2001b) Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. *Appl. Environ. Microbiol.* 67, 646-653.

Nykänen, A., Weckman, K. and Lapveteläinen, A. (2000) Synergistic inhibition of *Listeria monocytogenes* on cold-smoked rainbow trout by nisin and sodium lactate. *Int. J. Food Microbiol.* 61, 63-72.

Ouattara, B., Simard, R. E., Piette, G., Bégin, A. and Holley, R. A. (2000) Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int. J. Food Microbiol.* 62, 139-148.

Palou, E., Lopez-Malo, A., Barbosa-Canovas, G. V., Welti-Chanes, J. and Swanson, B. G. (1997) High hydrostatic pressure as a hurdle for *Zygosaccharomyces bailii* inactivation. *J. Food Sci.* 62, 855-857.

Papineau, A. M., Hoover, D. G., Knorr, D. and Farkas, D. F. (1991) Antimicrobial effect of watersoluble chitosans with high hydrostatic pressure. *Food Biotechnol.* 5, 45-57.

Park, S., Stan, S. D., Daeschel, M. A. and Zhao, Y. (2005) Antifungal coatings on fresh strawberries (*Fragaria ananassa*) to control mold growth during cold storage. *J. Food Sci.* 70, 202-207.

Pawar, D. D., Malik, S. V. S., Bhilegaonkar, K. N. and Barbuddhe, S. B. (2000) Effect of nisin and its combination with sodium chloride on the survival of *Listeria monocytogenes* added to raw buffalo meat mince. *Meat Sci.* 56, 215-219.

Pelroy, G. A., Peterson, M. E., Holland, P. J. and Eklund, M. W. (1994) Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium lactate. *J. Food Prot.* 57, 108-113.

Pen, L. T. and Jiang, Y. M. (2003) Effects of chitosan coating on shelf life and quality of fresh-cut Chinese water chestnut. *Lebensm. Wiss. Technol.* 36, 359–64.

Peng, C. H., Wang, Y. T. and Tang, Y. R. (1998) Synthesis of crosslinked chitosan crown ethers and evaluation of these products as adsorbents for metal ions. *J. Appl. Polym. Sci.* 70, 501-506.

Pine, L., Malcolm, G. B., Brooks, J. B. and Daneshvar, M. I. (1989) Physiological studies on the growth and utilization of sugars by *Listeria* species. *Can. J. Microbiol.* 35, 245-254.

Pirie, J. H. H. (1927) A new disease of veldt rodents, "Tiger River Disease". *S. Afr. Inst. Med. Res.* 3, 163-186.

Pranoto, Y., Rakshit, S. K. and Salokhe, V. M. (2005) Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT - Food Sci.Technol.* 38, 859-865.

Quintavalla, S. and Vicini, L. (2002) Antimicrobial food packaging in meat industry. *Meat Sci.* 62, 373-380.

Qvist, S., Sehested, K. and Zeuthen, P. (1994) Growth suppression of *Listeria monocytogenes* in a meat product. *Int. J. Food Microbiol.* 24, 283-293.

Ravi Kumar, M. N. V. (2000) A review of chitin and chitosan applications. *React. Funct. Poly.* 46, 1-27.

Ray, B. (1992) Nisin of *Lactococcus lactis* ssp. *lactis* as a food biopreservative. In Food origins (Eds. Ray, B. and Daeschel, M.). pp. 207-264. Boca Raton, FL. CRC Press, Inc.

Reddy, M. V. B., Belkacemi, K., Corcuff, R., Castaigne, F. and Arul, J. (2000) Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit. *Postharvest Biol. Technol.*20, 39-51.

Rhoades, J. and Roller, S. (2000) Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Appl. Environ. Microbiol.* 66, 80-86.

Roberts, G. A. F. (1992) Chitin chemistry. indianapolis, USA: Macmillan; 1992. pp274-301. Indianapolis, USA. Macmillan.

Rocourt, J. and Bille, J. (1997) Foodborne listeriosis. *Word Health Statistics Quarterly.* 50, 67-73.

Roller, S. and Covill, N. (2000) The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimp salads. *J. Food Prot.* 63, 202-209.

Romick, T. L., Fleming, H. P. and McFeeters, R. F. (1996) Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. *Appl. Environ. Microbiol.* 62, 304-307.

Rorvik, L. M., Aase, B., Alvestad, T. and Caugant, D. A. (2000) Molecular epidemiological survey of *Listeria monocytogenes* in seafoods and seafood-processing plants. *Appl. Environ. Microbiol.* 66, 4779-4784.

Rorvik, L. M. and Yndestad, M. (1991) *Listeria monocytogenes* in foods in Norway. *Int. J. Food Microbiol.* 13, 97-104.

Rozbeh, M., Kalchayanand, N., FIield, R. A., Johnson, M. C. and Ray, B. (1993) The influence of biopreservatives on the bacterial level of refrigerated vacuum packaged beef. *J. Food Saf.* 13, 99-111.

Ryser, E. T. and Marth, E. H. (2007) *Listeria,* listeriosis, and food safety, 3rd ed. Boca Raton, FL. CRC Press.

Ryser, E. T. and Marth, E. H. (1988) Survival of *Listeria monocytogenes* in cold-pack cheese food during refrigerated storage. *J. Food Prot.* 51, 615.

Ryu, C. H., Igimi, S., Inoue, S. and Kumagai, S. (1992) The incidence of *Listeria* species in retail foods in Japan. *Int. J. Food Microbiol.* 16, 157-160.

Sagoo, S., Board, R. and Roller, S. (2002) Chitosan inhibits growth of spoilage micro-organisms in chilled pork products. *Food Microbiol.* 19, 175-182.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A. and Smith, G. C. (2002) Control of *Listeria monocytogenes* with combined antimicrobials after postprocess contamination and extended storage of frankfurters at 4 degrees C in vacuum packages. *J. Food Prot.* 65, 299-307.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A., & Smith, G. C. (2005) Combinations of nisin with organic acids or salts to control *Listeria monocytogenes* on sliced pork bologna stored at 4°C in vacuum packages. *LWT.* 38, 21-28.

Samelis, J., Sofos, J. N., Kain, M. L., Scanga, J. A., Belk, K. E. and Smith, G. C. (2001) Organic acids and their salts as dipping solutions to control *Listeria monocytogenes* inoculated following processing of sliced pork bologna stored at 4 degrees C in vacuum packages. *J. Food Prot.* 64, 1722-1729.

Schlyter, J. H., Glass, K. A., Loeffelholz, J., Degnan, A. J. and Luchansky, J. B. (1993a) The effects of diacetate with nitrite, lactate, or pediocin on the viability of *Listeria monocytogenes* in turkey slurries. *Int. J. Food Microbiol.* 19, 271-281.

Schlyter, J. H., Degnan, A. J., Loeffelholz, J., Glass, K. A. and Luchansky, J. B. (1993b) Evaluation of sodium diacetate and ALTA 2341 on viability of *Listeria monocytogenes* in turkey slurries. *J. Food Prot.* 56, 808-810.

Seeliger, H. P. R. and Bockemuhl, J. (1968) Kritische Untersuchungen zur Frage einer kapselbildung bei *Listeria monocytogenes*. *Zbl. Bakteriol. Parasit. Infekt. Hyg. I. Orig.* 206, 216-227.

Shahidi, F., Arachchi, J. K.V. and Jeon, Y. J. (1999) Food applications of chitin and chitosans. *Trends Food Sci. Technol.* 10, 37-51.

Shelef, L. A. (1994) Antimicrobial effects of lactates: A review. *J. Food Prot.* 57, 445-450.

Shelef, L. A. and Addala, L. (1994) Inhibition of *Listeria monocytogenes* and other bacteria by sodium diacetate. *J.Food Saf.* 14, 103-115.

Shelef, L. A. and Yang, Q. (1991) Growth suppression of *Listeria monocytogenes* by lactates in broth, chicken, and beef. *J. Food Prot.* 54, 283-287.

Sofos, J. N. (1989) Sorbate food preservatives. Boca Raton, FL. CRC Press.

Sofos, J. N. and Busta, F. F. (1993) Sorbic acid and sorbates. In Antimicrobials in foods (Eds Davidson P.M. and Branen, A.L.) pp. 49-94. New York. Marcel Dekker.

Stekelenburg, F. K. and Kant-Muermans, M. L. T. (2001) Effects of sodium lactate and other additives in a cooked ham product on sensory quality and development of a strain of *Lactobacillus curvatus* and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 66, 197-203.

Stillmunkes, A. A., Prabhu, G. A., Sebranek, J. G. and Molins, R. A. (1993) Microbiological safety of cooked beef roasts treated with lactate, monolaurin or gluconate. *J. Food Sci.* 58, 953-958.

Sudarshan, N. R., Hoover, D. G. and Knorr, D. (1992) Antibacterial action of chitosan. *Food Biotechnol.* 6, 257-272.

Sugano, M., Yoshida, K., Hashimoto, M., Enomoto, K. and Hirano, S. (1992) Hypocholesterolemic activity of partially hydrolyzed chitosan in rats. In Advances in chitin and chitosan (Eds. Brine, C. J., Sandford, P. A. and Zikakis, J. P.). pp. 472-478. London, U.K. Elsevier.

Tokoro, A., Tatewaki, N., Suzuki, K., Mikami, T., Suzuki, S. and Suzuki, M. (1988) Growth-inhibitory effect of hexa-*N*-acetylchitohexaose and chitohexaose against meth-A solid tumor. *Chem Pharm Bull.* 36, 786-790.

Tompkin, R. B. (2002) Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65, 709-725.

Torres, J. A., Motoki, M. and Karel, M. (1985) Microbial stabilization of intermediate moisture food surfaces. I. Control of surface preservative concentration. *J. Food Process. Preserv.* 9, 75-92.

Tsai, G. J. and Su, W. H. (1999) Antibacterial activity of shrimp chitosan against *Escherichia coli. J. Food Prot.* 62, 239-243.

Tsai, G. J., Wu, Z. Y., Chen, H. C. and Pan, C. L. (2002) Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. *Fisheries Sci.* 68, 170-177.

Tsai, G. J., Wu, Z. Y. and Su, W. H. (2000) Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. *J. Food Prot.* 63, 747-752.

Uchida,Y., Izume, M. and Ohtakara, A. (1989) Preparation of chitosan oligomers with purified chitosanase and its application. In Chitin and chitosan: Sources, chemistry, biochemistry, physical properties and applications (Eds. Skjåk-Bræk, G., Anthonsen, T. and Sandford, P.). pp. 373-382. London, U.K. Elsevier.

Uhart, M., Ravishankar, S. and Maks, N. D. (2004) Control of *Listeria monocytogenes* with combined antimicrobials on beef franks stored at 4°C. *J. Food Prot.* 67, 2296-2301.

Unda, J. R., Molins, R. A. and Walker, H. W. (1991) *Clostridium sporogenes* and *Listeria monocytogenes*: Survival and inhibition in microwave-ready beef roasts containing selected antimicrobials. *J. Food Sci.* 56, 198-205.

USDA-FSIS. (2003) *Listeria* in FSIS ready-to-eat products shows significant decline. Washington, DC. USDA-FSIS.

Vargas, M., Albors, A., Chiralt, A. and Gonzalez-Martinez, C. (2006) Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings. *Postharvest Biol. Technol.*41, 164-171.

Vartiainen, J., Skytta, E., Enqvist, J. and Ahvenainen, R. (2003) Properties of antimicrobial plastics containing traditional food preservatives. *Packag. Technol. Sci.* 16, 223-229.

Vitas, A. I., Aguado, V. and Garcia-Jalon, I. (2004) Occurrence of *Listeria monocytogenes* in fresh and processed foods in Navarra (Spain). *Int. J. Food Microbiol.* 90, 349-356.

Vogel, B. F., Huss, H. H., Ojeniyi, B., Ahrens, P. and Gram, L. (2001) Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing

plants detected by DNA-based typing methods. *Appl. Environ. Microbiol.* 67, 2586-2595.

Vojdani, F. and Torres, J. A. (1990) Potassium sorbate permeability of methylcellulose and hydroxypropyl methylcellulose coatings: Effect of fatty acids. *J. Food Sci.* 55, 841-846.

Vorlop, K. D. and Klein, J. (1981) Formation of spherical chitosan biocatalysts by ionotropic gelation. *Biotechnol. Lett.* 3, 9-14.

Wang, G. H. (1992) Inhibition and inactivation of five species of foodborne pathogens by chitosan. *J. Food Prot.* 55, 916-919.

Weiner, M. L. (1992) An overview of the regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredients. In Advances in chitin and chitosan (Eds. Brine, C. J., Sandford, P. A. and Zikakis, J. P.). pp. 663-670. London, U.K. Elsevier.

Wilson, I. G. (1995) Occurrence of *Listeria* species in ready to eat foods. *Epidemiol. Infect.* 115, 519-526.

Wit, J. C. and Rombouts, F. M. (1990) Antimicrobial activity of sodium lactate. *Food Microbiol.* 7, 113-120.

Wong, D. W. S., Gastineau, F. A., Gregorski, K. S., Tillin, S. J. and Pavlath, A. E. (1992) Chitosan-lipid films: Microstructure and surface energy. *J. Agric. Food Chem.* 40, 540-544.

Yoon, K. S., Burnette, C. N., Abou-Zeid, K. A.and Whiting, R. C. (2004) Control of growth and survival of *Listeria monocytogenes* on smoked salmon by combined potassium lactate and sodium diacetate and freezing stress during refrigeration and frozen storage. *J. Food Prot.* 67, 2465-2471.

Yoon, K. S., Burnette, C. N. and Whiting, R. C. (2003) Effects of pH and agitation on the growth of *Listeria monocytogenes* Scott A in brain heart infusion broth containing combined potassium lactate and sodium diacetate during storage at 4 or 10°C. *J. Food Prot.* 66, 1469-1473.

Young, D. H., Kohle, H.and Kauss, H. (1982) Effect of chitosan on membrane permeability of suspension-cultured glycine max and *Phaseolus vulgaris* cells. *Plant Physiol.* 70, 1449-1454.

Yousef, A. E., El-Shenawy, M. A.and Marth, E. H. (1989) Inactivation and injury of *Listeria monocytogenes* in a minimal medium as affected by benzoic acid and incubation temperature. *J. Food Sci.* 54, 650-652.

Zhang, D. and Quantick, P. C. (1997) Effects of chitosan coating on enzymatic browning and decay during postharvest storage of litchi (*Litchi chinensis sonn.*) fruit. *Postharvest Biol. Technol.*12, 195-202.

Zhu, M. J., Mendonca, A., Ismail, H. A., Du, M., Lee, E. J. and Ahn, D. U. (2005) Impact of antimicrobial ingredients and irradiation on the survival of *Listeria monocytogenes* and the quality of ready-to eat turkey ham. *Poultry Sci.* 84, 613-620.

Zivanovic, S., Draughon, A. F.and Chi, S. (2005) Antimicrobial activity of chitosan films enriched with essential oils. *J. Food Sci.* 70, 45-51.

Chapter 3

CONTROL OF *LISTERIA MONOCYTOGENES* ON HAM STEAKS BY ANTIMICROBIALS INCORPORATED INTO CHITOSAN-COATED PLASTIC FILMS

Abstract

Contamination of ready-to-eat (RTE) meat products such as ham steaks with *Listeria monocytogenes* has been a concern for the meat processing industry. The objective of this study was to evaluate the antilisterial efficacy of chitosan-coated plastic films alone or incorporating five Generally Recognized as Safe (GRAS) antimicrobials. Effect of chitosan-coated plastic film on the growth of *L. monocytogenes* was first investigated in an aqueous system of culture medium broth and chitosan-coated films were able to inhibit the growth of *L. monocytogenes* in a concentration-dependent manner. However, chitosan-coated plastic films were not able to control the growth of *L. monocytogenes* on ham steaks. Therefore, five GRAS antimicrobials were subsequently incorporated into chitosan-coated plastic films to enhance their antilisterial effectiveness. Ham steaks were surface-inoculated with a five-strain cocktail of *L. monocytogenes* and then packaged in chitosan-coated plastic

films containing 500 IU/cm^2 of nisin, 10 mg/cm^2 of sodium lactate (SL), 2.5 mg/ cm^2 of sodium diacetate, 3 mg/cm^2 of potassium sorbate, or 1 mg/cm^2 of sodium benzoate. The samples were stored at room temperature (ca. 20°C) for 10 days. Incorporating antimicrobials into chitosan-coated plastic films slowed down or inhibited the growth of *L. monocytogenes*. The chitosan-coated plastic film containing SL was the most effective antimicrobial film and its efficacy against *L. monocytogenes* on ham steaks was evaluated during 12-week storage at 4°C. The film showed excellent long-term antilisterial effect with the counts of *L. monocytogenes* being slightly lower than the initial inoculum. Chitosan-coated plastic films containing 10 mg/cm^2 of SL have a potential to be used on ham steaks to control *L. monocytogenes*.

3.1. Introduction

Listeria monocytogenes is a foodborne pathogen of particular concern in ready-to-eat (RTE) meat products because of its ability to survive and grow at refrigeration temperatures, its capacity to tolerate relatively high heat and high concentrations of salt and the high fatality rate associated with listeriosis. *L. monocytogenes* has been involved in numerous foodborne illness outbreaks associated with RTE meats. In 1998 and 1999, a significant outbreak occurred with frankfurters, which resulted in 21 deaths and approximately 100 reported cases of listeriosis (Centers for Disease Control and Prevention (CDC), 1999). Another notable outbreak occurred in the northeastern United States in 2002, resulting in 10 fatalities associated with the consumption of sliced turkey deli meat (CDC, 2000; 2002). *L. monocytogenes*

accounts for 28% of the deaths resulting from foodborne illnesses in the United States, which is second only to *Salmonella* (31%) (Mead et al., 1999).

L. monocytogenes is a frequent surface contaminant of RTE meats often occurring during the post-processing phase (Tompkin, 2002). Novel means to control this contamination have been sought (Wakabayashi et al., 1992). Antimicrobial packaging can be a promising tool for protecting RTE meats from *L. monocytogenes* contamination (Janes et al., 2002; Lungu and Johnson, 2005). Antimicrobial packaging films act by preventing microbial growth on a food surface by direct contact of the package with the surface of food. The gradual release of an antimicrobial substance from a packaging film to the food surface for extended period of time may be more advantageous than incorporating the antimicrobial into foods. In the latter processes, antimicrobial activity may be lost or reduced due to inactivation of the antimicrobial compound by food components (Appendini and Hotchkiss, 2002; Quintavalla and Vicini, 2002).

Chitosan is a natural polymer obtained by deacetylation of chitin, which is the major constituent of the exoskeleton of crustaceans. Chitosan has been proved to be nontoxic, biodegradable, and biocompatible. Chitosan is insoluble in water, but soluble in various acidic solvents such as dilute hydrochloric, formic and acetic acids. In an acidic solution, the amine groups on the chitosan molecule are protonated to NH_3^+ and thus acquire a positive charge (Shahidi et al., 1999; Ravi Kumar, 2000). Chitosan has intrinsic antimicrobial activity and inhibits the growth of a wide variety

of bacteria (Shahidi et al., 1999; Helander et al., 2001). In this study chitosan was used as a carrier for antimicrobials. Since edible film formed by chitosan is brittle and does not have good mechanical properties, in this study chitosan was coated onto a plastic film to overcome these shortcomings. The additional benefit of coating chitosan onto plastic films is that chitosan is not consumed with food. Using edible coatings to carry functional substances is not a new concept. Plastic films coated with edible coatings carrying spices and flavoring substances are commercially used to transfer those substances to the surfaces of meat, poultry and fish products.

To enhance the efficacy of chitosan-coated film against *L. monocytogenes*, five Generally Recognized as Safe (GRAS) antimicrobials, nisin, sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PS), and sodium benzoate (SB), were incorporated into the chitosan coating in this study. It has been well documented that these antimicrobials can control *L. monocytogenes* in meat products (Samelis et al., 2002; Szabo and Cahill, 1999; Glass et al., 2002； Schlyter et al., 1993a, b; Lu et al., 2005; Islam et al., 2002). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, exerts rapid bactericidal effects against gram-positive bacteria, especially against strains of *L. monocytogenes*, in laboratory media or model food systems (Delves-Broughton and Gasson, 1994). SL is primarily used as a flavor enhancer in meat and poultry products (Shelef, 1994). SD is a derivative of acetic acid and is used in bread and cakes to prevent mold growth (Jay, 2000). At 0.1 to 0.3%, SD can control growth of *L. monocytogenes* in meat (Schlyter et al., 1993; Ghanem and Skonberg,

2002). PS is primarily used to control yeasts and molds. Effective antimicrobial concentrations of PS in most foods are in the range of 0.05 to 0.30 % (Sofos and Busta, 1993). El-Shenawy and Marth (1988) found that PS inhibited or inactivated *L. monocytogenes* in a broth substrate, depending on pH and concentration. The antibacterial properties of sodium benzoate (SB) are due to the undissociated, molecular form of benzoic acid (Doores, 1993). These studies have investigated the efficacy of these antimicrobials when directly added into or onto food products. An alternative way of controlling *L. monocytogenes* is through their incorporation into a packaging material that is subsequently applied onto food. To our knowledge, no research has been conducted to compare the effectiveness of these five antimicrobials incorporated into packaging films for controlling *L. monocytogenes*.

The objectives of this study were to evaluate the efficacy of chitosan-coated plastic films and chitosan-coated plastic films incorporating antimicrobials on controlling the growth of *L. monocytogenes* on ham steaks. The effectiveness of these antimicrobial packaging films was evaluated at room temperature. The most effective antimicrobial film was selected and its efficacy against *L. monocytogenes* on ham steaks was assessed at refrigeration temperature (4°C) for 12 weeks.

3.2. Materials and Methods

3.2.1. Effect of chitosan-coated plastic film on the growth of *L. monocytogenes* in a culture medium broth

3.2.1.1 Coating of plastic film with chitosan.

Two grams of medium molecular weight (MMW) chitosan (Sigma-Aldrich, St. Louis, MO) were dissolved in 100 ml of 1% (w/v) acetic acid and stirred overnight at room temperature (chitosan concentration = 0.02 g/ml or 2%). Hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich) solution was prepared by dissolving 3 g of HPMC and 0.33 ml of polyethylene glycol 400 (Fisher Scientific, Hampton, NH) in 100 ml of 1% acetic acid. The coating solution of the first layer was prepared by mixing equal volumes of the chitosan and HPMC solutions according to the method described by Moller et al. (2004).

A Surlyn® film (2.0 mil) was taped to 20 x 20 cm glass plates and 15 ml of the chitosan-HPMC coating solution was cast onto the plastic film using a thin-layer chromatography plate coater (TLC, CAMAG, Muttenz, Switzerland). The gate of the TLC coater was fixed at 500 μm to control the thickness of the coating. The coated film was air-dried at room temperature overnight. Then 15 ml of the chitosan solution was cast onto the first layer of coating and air-dried overnight to form a second layer and the same procedure was repeated for the third layer using the same volume of chitosan solution. It was found that without incorporating HPMC, the first chitosan layer could not be coated uniformly onto the plastic film. The chitosan-coated film contained 2.5 mg of chitosan per cm^2 of film surface. A control film was made by coating a Surlyn® film with the HPMC solution. The films were subsequently cut into discs with diameter of 1.2 cm and UV-treated for 2 minutes to sterilize the films.

3.2.1.2 Indicator microorganism.

L. monocytogenes ATCC 19115 was maintained on tryptic soy agar plus 0.6% yeast extract (TSAYE) (Difco Laboratories, Detroit, MI) agar plates at 4°C. The culture was transferred monthly onto a freshly made TSAYE agar plate during the experimental period. For growth, a single colony of *L. monocytogenes* was inoculated into a tube of tryptic soy broth plus 0.6% yeast extract (TSBYE) (Difco Laboratories) broth and incubated at 35°C for 24 h. The culture was then transferred to 10 ml of fresh TSBYE and incubated for 24 h at 35°C to reach a final concentration of approximately 10^9 CFU/ml.

3.2.1.3. Shake flask assay.

Two, 4, 8, 16, and 32 discs of chitosan-coated film were placed into tubes containing 10 ml of TSBYE and the tubes of TSBYE were immediately inoculated with the *L. monocytogenes* culture prepared above to a final concentration of 10^5 CFU/ml. A control was prepared by placing 32 discs of the control film in 10 ml of TSBYE and then inoculating the TSBYE with 10^5 CFU/ml of *L. monocytogenes*. The TSBYE tubes were then incubated at 35ºC for 72 h with shaking at 100 rpm. Counts of *L. monocytogenes* in the TSBYE tubes were determined at 8, 16, 24, 48, and 72 h by plating samples onto TSAYE plates and incubating the plates at 35ºC for 2 days.

3.2.1.4. Comparison of the antilisterial activity of low and medium molecular weight chitosans

Since the molecular weight of chitosan affects its antimicrobial activity (No et al., 2002), the antilisterial activity of two chitosans, low molecular weight (LMW) (Sigma-Aldrich) and MMW chitosans, were compared. Surlyn® films were coated with LMW chitosan or MMW chitosan to a concentration of 2.5 mg/cm^2. Eight and 16 discs of LMW or MMW chitosan-coated film were tested using the shake flask assay as described above. Based on the counts of *L. monocytogenes*, LMW chitosan was more effective in inhibiting the growth of *L. monocytogenes* than MMW chitosan and was thus used in the following experiments.

3.2.2. Effect of chitosan-coated film on the growth of *L. monocytogenes* on ham steaks

3.2.2.1. Selection of a most chitosan-resistant strain of *L. monocytogenes*.

It is logical to use a most chitosan-resistant strain in a study to represent a worst case scenario. Twelve strains of *L. monocytogenes* were used in this study. The strains were ATCC 19115, ATCC 19113, CCR8, CA, F5069, V7, PSU1, PSU2, PSU9, PSU21, PSU23, and Scott A that were kindly provided by Drs. Hoover and Joerger at the University of Delaware and Dr. Knabel at the Pennsylvania State University. The individual cultures of *L. monocytogenes* were prepared as described above. The cultures were diluted in fresh TSBYE to a final concentration of 10^5 CFU/ml of *L. monocytogenes*. Each strain of *L. monocytogenes* in 10-ml TSBYE was incubated with 0 (control) and 8 discs of the chitosan-coated plastic film at 35ºC with shaking at 100 rpm. Counts of *L. monocytogenes* were determined at 24 and 48 h by plating samples

onto TSAYE plates and incubating the plates at 35ºC for 2 days. The F5069 strain which consistently had the highest counts among 12 strains was found to be most resistant to chitosan.

3.2.2.2. Inoculation of ham steak samples.

Freshly processed ham steak samples were obtained from a local retailer. They were kept frozen at -20°C and thawed at 2°C for 1 day immediately before use as described by Besse et al. (2004). Slices of ham steak were punched aseptically into 5.7-cm diameter round pieces weighing 28±1 g with a surface area of 25.7 cm^2 on one side. The ham steak discs were placed onto a piece of sterile aluminum foil. The culture of *L. monocytogenes* F5069 was prepared as described above and 0.125 ml of this culture was spread on one side of the ham steak surfaces, and the samples were left undisturbed for 5 min to allow the inoculum to soak in and the cells to attach. The same procedure was repeated on the other side of each sample thus achieving a final concentration of 5 x 10^3 CFU/cm^2 of ham steak surface. After inoculation, ham steak samples were kept at room temperature for 20 min to allow cell attachment.

3.2.2.3. Packaging of inoculated ham steak samples with chitosan-coated plastic film.

The inoculated samples were wrapped in the chitosan-coated Surlyn® films containing 2.5 or 5.5 mg of chitosan per cm^2 of film surface and a plain Surlyn® film without chitosan coating (control). The wrapped samples were then inserted into 3-mm thick high barrier pouches (nylon/polyethylene, Koch Supplies, Kansas City,

MO) and subsequently sealed using a vacuum-packaging machine (Model Ultravac 225 with digital control panel, Koch Equipment, Kansas City, MO). The packages were stored at 20°C for 10 days. Instead of using 4°C, a typical refrigerated storage temperature for this kind of products, a relative high temperature was used so that the efficacy of chitosan against *L. monocytogenes* could be determined in a relatively shorter period of time.

3.2.2.4. Analysis of *L. monocytogenes* in the samples.

The samples were analyzed for *L. monocytogenes* every 2 days over the storage period. For determination of *L. monocytogenes* counts, a package was opened aseptically and the contents were transferred to a sterile stomacher bag and homogenized for 2 min with 100 ml of 0.1% peptone water. Ten-fold serial dilutions were made using 0.1% peptone water. Counts of *L. monocytogenes* were determined by an overlay method (Kang and Fung, 1999). Briefly, the serial dilutions were spread-plated onto solidified TSAYE agar plates and the plates were incubated at 35°C for 3 h. Approximately 7 ml of modified Oxford medium (Difco Laboratories) at 45°C was overlaid on the TSAYE plates. The plates were incubated at 35°C for 48 h and small black colonies with black haloes on the plates were counted. Occasionally, colonies were confirmed to be *L. monocytogenes* using a BAX™ for Screening/*Listeria monocytogenes* PCR assay (Qualicon-DuPont, Wilmington, DE). The numbers of *L. monocytogenes* per cm^2 were calculated by dividing the total count of *L. monocytogenes* per ham steak disc by the total surface area (51.4 cm^2).

3.2.3. Screening chitosan-coated plastic films incorporating various GRAS antimicrobials for inhibiting the growth of *L. monocytogenes* on ham steaks.

3.2.3.1. Preparation of antimicrobial films.

Five antimicrobials, nisin (Sigma-Aldrich), SD (Sigma-Aldrich), SL (Fisher), SB (Fisher), and PS (Fisher), were incorporated into the chitosan solution. The antimicrobial coating solutions were prepared by mixing 0.2 g of nisin, 1.0 g of SD, 6.7 g of a 60% SL syrup, 0.4 g of SB, and 1.2 g of PS, with 15 ml of the 2% chitosan solution. The antimicrobial coating solutions were cast onto Surlyn® films using the TLC coater. The coated films contained 1 mg/cm^2 of chitosan incorporating 500 IU/cm^2 of nisin, 10 mg/cm^2 of SL, 2.5 mg/ cm^2 of SD, 3 mg/cm^2 of PS, or 1mg/cm^2 of SB. A relatively low level of chitosan, 1 mg/cm^2 instead of 2.5 mg/cm^2, was used for the coatings since chitosan alone was not effective against *L. monocytogenes* on ham steaks. A plain Surlyn® film and a film coated with 1 mg/cm^2 of chitosan were used as controls. The coated films were air-dried at room temperature overnight.

3.2.3.2. Inoculation of ham steak samples.

A cocktail of five strains of *L. monocytogenes* was used to increase genetic variability. These strains included two nisin-resistant strains, PSU1 (Serotype 1/2a) and PSU21 (Serotype 4b), the chitosan-resistant strain, F5069 (Serotype 4b), and two other strains, PSU9 (Serotype 1/2b) and Scott A (Serotype 4b). The cultures of the five strains of *L. monocytogenes* were prepared as described above. A 1-ml volume of

each culture was pooled to provide the cocktail. Discs of ham steak samples were prepared and inoculated with the cocktail as described above.

3.2.3.3. Packaging of inoculated ham steak samples with antimicrobial films.

The inoculated samples were wrapped in the antimicrobial and control films prepared above. The wrapped samples were then inserted into the high barrier pouches and vacuum packaged. The packages were stored at 20°C for 10 days. The counts of *L. monocytogenes* in the ham steak samples were determined using the overlay method every 2 days. The chitosan-coated film incorporating SL was found to be most effective and used in the following refrigerated storage study.

3.2.4. Refrigerated storage study

Ham steak samples were surface-inoculated with the five-strain cocktail of *L. monocytogenes* to a final concentration of approximately 5 x 10^2 CFU/cm^2. The samples were then wrapped with chitosan-coated film containing 10 mg /cm^2 of SL, placed in the high barrier pouches and vacuum packaged. A plain Surlyn® film and a film coated with 1 mg/cm^2 chitosan were used as controls. Samples were stored at 4°C for 12 weeks and counts of *L. monocytogenes* were analyzed weekly. Total aerobic and anaerobic counts in un-inoculated ham steak samples were also determined using TSAYE and anaerobic agar (Difco Laboratories), respectively. Plates were incubated at 35°C for 2 days. Presence of *L. monocytogenes* in the un-inoculated ham steak samples was determined by a primary enrichment in UVM broth (Difco Laboratories) and a secondary enrichment in Fraser broth (Difco Laboratories) according to the

USDA Microbiology Laboratory Guidebook (USDA-FSIS, 2006) at the beginning of the experiment.

3.2.5. Statistical analysis

Three independent trials were conducted for each experiment. Statistical analysis was conducted using Microsoft® Office Excel 2003. One-way analysis of variance (ANOVA) was used to compare significant differences between treatments ($P < 0.05$).

3.3. Results and Discussion

3.3.1. Effect of chitosan-coated plastic film on the growth of *L. monocytogenes* in a culture medium broth

Figure 1 shows the effect of chitosan-coated films on the growth of *L. monocytogenes* in TSBYE broth. To make the figures easy to read, error bars are not shown in this figure. The average standard deviation for all the data points was 0.5 log CFU/ml. In the control sample, 32 discs of HPMC-coated Surlyn® film in TSBYE, *L. monocytogenes* grew rather rapidly, reaching approximately 10^8 CFU/ml in 8 h and 10^9 CFU/ml in 16 h. Chitosan-coated films considerably inhibited the growth of *L. monocytogenes*. Addition of 2 discs of chitosan-coated film to the TSBYE culture reduced the counts of *L. monocytogenes* to approximately 10^4 CFU/ml in 8 h. *L. monocytogenes* then started to grow at a rate similar to the control sample, but to final levels lower than the control sample. Increasing the number of discs (or increasing the concentrations of chitosan) in the TSBYE culture increased the inhibition of *L.*

monocytogenes. Addition of 32 discs of chitosan-coated film reduced the counts of *L. monocytogenes* to below detection limit, 1 CFU/ml, after 72 h incubation at 35°C. These results are in agreement with those reported by Zivanovic et al. (2004). They found that during storage at 25°C, emulsions with 0.58% acetic acid and 0.1% chitosan reduced the initial inoculum of *L. monocytogenes* from a level of 10^7 CFU/ml to below detection limits after 24, 48, 72, or 96 h.

The effect of MMW and LMW chitosan-coated films on the growth of *L. monocytogenes* in TSBYE was compared (Figure 2). For the 8-disc treatment, there were no significant differences in the counts of *L. monocytogenes* between the MMW and LMW chitosans at 8, 16, 48, and 72 h ($P > 0.05$). Although not significant different ($P = 0.052$), LMW chitosan consistently resulted in lower counts of *L. monocytogenes* than MMW chitosan for all the replicates conducted at 72 h and the difference in counts between the two chitosans was 1.8 log CFU/ml. At 24 h, MMW chitosan was more effective against *L. monocytogenes* than LMW chitosan ($P < 0.05$) and the difference in counts between the two chitosans was 1.5 log CFU/ml. For the 16-disc treatment, there were no significant differences in the counts of *L. monocytogenes* between the MMW and LMW chitosans at 8, 24, and 48 h ($P > 0.05$). At 16 and 72 h, LMW chitosan was more effective against *L. monocytogenes* than MMW chitosan ($P < 0.05$). Overall, LMW chitosan was more effective than MMW chitosan at the end of the experiment period and thus it was chosen to be used in the following experiments.

The antibacterial effect of chitosan and its oligomers is reported to be dependent on their molecular weight (Jeon et al., 2001; No et al., 2002). Liu et al. (2001) found that the antimicrobial activity increased with molecular weight of chitosan from 5.0 x 10^3 to 9.2 x 10^4 Da, but then decreased when the molecular weight further increased to 1.1 x 10^6 Da. Jeon et al. (2001) compared chitosan with an average molecular weight of 6.9 x 10^5 Da to oligomers with high (7 to 24 kDa), medium (1.5 to 6 kDa), and low (1 to 1.5 kDa) molecular weights. They found that chitosan with a minimum molecular weight of 10 kDa was required to inhibit both gram-positive and gram-negative bacteria. In this study, the LMW chitosan-coated film was more effective against *L. monocytogenes* than the MMW chitosan-coated film. This difference might be due to the effectiveness of these two chitosans themselves. It is also possible that LMW chitosan has a faster diffusion rate and higher solubility in TSBYE than MMW chitosan because of its low molecular size.

3.3.2. Effect of chitosan-coated film on the growth of *L. monocytogenes* on ham steaks

Figure 3 shows the responses of 12 strains of *L. monocytogenes* to chitosan. After incubation with chitosan-coated film discs in TSBYE broth for 24 and 48 hours, strain F5069 resulted in 1.0 and 1.7 log CFU/ml reductions, respectively, which were significantly lower than the other 11 strains ($P < 0.05$). To our knowledge, this is the first study demonstrating that there are variations in sensitivity to chitosan among strains of *L. monocytogenes*. It is well documented that different strains of *L.*

monocytogenes have different resistances to a variety of antimicrobial agents (Prazak et al., 2002). For example, variation in resistance to nisin among strains of *L. monocytogenes* has been demonstrated (Benkerroum and Sandine, 1988; Harris et al., 1991; Ukuku and Shelef, 1997).

Since strain F5069 had the highest resistance to chitosan among the 12 strains, it was used to inoculate ham steak samples in this part of the study to represent a worst case scenario. Counts of *L. monocytogenes* on ham steak samples packaged in plain and chitosan-coated Surlyn® films and stored at 20°C are shown in Figure 4. Plain Surlyn® film allowed *L. monocytogenes* on ham steaks to grow rapidly. The population of *L. monocytogenes* reached > 7.0 log CFU/cm^2 after 4 days at room temperature. The growth curves of plastic films coated with 2.5 and 5.5 mg/cm^2 of chitosan were similar to the plain film although the counts of *L. monocytogenes* in these two treated samples were consistently lower than those in the plain film sample at all time points. The difference in counts between the two chitosan treatments and the control for all the data points were ≤ 0.7 log CFU/cm^2. In addition, these differences in counts were not significant ($P > 0.05$). Hence it was concluded that chitosan-coated plastic films did not inhibit the growth of *L. monocytogenes* or its inhibition effect was minimal. Therefore, chitosan-coated plastic film alone could not be used to control the growth of *L. monocytogenes* on ham steaks.

Chitosan has intrinsic antimicrobial activity, which is effectively expressed in aqueous systems (Sudarshan et al., 1992; Wang, 1992). However,

antimicrobial properties may become negligible when chitosan is in the form of insoluble films. This suggests that dispersion of the chitosan molecules within the meat matrix is a prerequisite for its antimicrobial action (Ouattara et al., 2000). Coma et al. (2001) found that chitosan was incapable of diffusing through a solid medium such as agar. The antilisterial effect of chitosan films were tested in this study using an inhibition zone assay. No inhibition zone was found when chitosan-coated plastic films were placed on agar medium inoculated with *L. monocytogenes* after 24 h of incubation at 35°C (data not shown). Hence it is possible that chitosan is ineffective in films because it is unable to diffuse through a rigid food matrix such as ham steaks.

3.3.3. Screening chitosan-coated plastic films incorporating GRAS antimicrobials for inhibiting the growth of *L. monocytogenes* on ham steaks

Figure 5 shows the effect of plastic films coated with chitosan containing GRAS antimicrobials on the growth of *L. monocytogenes* on ham steaks. To make the figure easy to read, error bars are not shown in this figure. The average standard deviation for all the data points was 0.3 log CFU/cm^2. The initial concentration of *L. monocytogenes* on inoculated ham steak samples was 5.7 log CFU/cm^2. *L. monocytogenes* in the two controls, plain and chitosan-coated films, grew to more than 7 log CFU/cm^2 after 10 days of storage at room temperature. Incorporating antimicrobials into chitosan slowed down or inhibited the growth of *L. monocytogenes*. The SL and PS were the most effective antimicrobials reducing the initial counts from 5.7 to ≤ 5.2 log CFU/cm^2 after 10 days of storage. The counts of *L. monocytogenes* in

these two treatments were more than 1.9 log CFU/cm^2 lower than those in the two controls after 10 days of storage. These differences in counts were statistically significant ($P < 0.05$). Moreover, the SL treatment consistently had lowest counts of *L. monocytogenes* than other treatments at all time points.

Compared to chitosan, the five antimicrobials used in this study are relatively small molecules. Nisin has a molecular weight of 3510 Da (Thomas and Delves-Broughton, 2005) and the other four antimicrobials used in this study are salts of organic acids which have very low molecular weights. These small molecules should be able to diffuse from the chitosan films to the ham steak samples. It has been demonstrated that nisin is able to diffuse from a methylcellulose/HPMC-coated plastic film to an agar medium (Neetoo et al., 2007). The diffusion of PS from edible films such as chitosan, methylcellulose and HPMC to the surface of food has also been demonstrated (Vojdani and Torres, 1989). The gradual release of these antimicrobials from the chitosan coating to the ham steaks surface may be more advantageous than incorporating them directly into ham steaks since the antimicrobials being carried are likely to remain at high concentrations for an extended period of time on the food surface (Coma et al., 2001).

The differences in the effectiveness of these antimicrobials might be due to the intrinsic properties of the antimicrobials themselves, the concentrations used, and the rate of diffusion of the antimicrobials to the ham steaks. Having an optimum diffusion rate is important; a too high diffusion rate would cause the antimicrobials to

migrate rapidly into the food matrix thereby reducing the antimicrobial concentrations at the ham steak surface to inactive levels while a too low diffusion rate might provide *L. monocytogenes* with sufficient time to grow to high levels. The concentrations of the antimicrobials were also important for the effectiveness of these antimicrobial films. The concentrations of SD, PS, and SB used in this study were at their maximum legal limits (Bedie et al., 2001; USDA-FSIS, 2000; Doores, 1993). Nisin has a legal limit of 10,000 IU/g (Hurst and Hoover, 1993). A much lower nisin level, 500 IU/cm^2, was used in this study since nisin is relatively expensive and the cost of using high nisin levels would be prohibitive for the food industry. SL was the most effective antimicrobial against *L. monocytogenes* and the concentration used in this study, 10 mg/cm^2 or ca. 1.8% (w/w), was much lower than its legal limit, 4.8% (w/w) (USDA-FSIS, 2000). Therefore, SL was used in the following study to determine whether SL was still effective against *L. monocytogenes* during long term refrigerated storage.

3.3.4. Refrigerated storage study

In the last phase of this study, chitosan-coated plastic film containing 10 mg/cm^2 of SL was selected in the investigation of its long term antilisterial effectiveness when used on ham steaks. Representative samples of ham steak had no detectable *Listeria* spp.; therefore all *Listeria* spp. found in the inoculated samples originated from the inoculum and was *L. monocytogenes*. The mean population of *L. monocytogenes* on inoculated treated sample as recovered just after inoculation was 2.7 log CFU/cm^2. *L. monocytogenes* cell counts on ham steak samples treated with

chitosan-based films incorporating SL are shown in Figure 6. In the first 5 weeks, *L. monocytogenes* in the two controls, plain film and film coated with chitosan, grew very slowly from 2.7 to 3.5 log CFU/cm^2. It started to grow rather rapidly from week 5 and reached 7.8 log CFU/cm^2 after 12 weeks storage at 4°C. The SL treatment was effective against *L. monocytogenes* and reduced its counts from 2.7 to 1.5 log CFU/cm^2 during 10 weeks of storage. *L. monocytogenes* started to grow on week 10, but the count of *L. monocytogenes* at the end of the 12-week storage was still slightly lower than the initial count. These results demonstrate that chitosan-coated plastic film incorporated with SL could be used to control *L. monocytogenes* on ham steaks. These results also suggest that the effectiveness of antimicrobials films could be tested at room temperature to considerably decrease the testing time since the results obtained from this part of the study were in agreement with those obtained in the screening study described above (Figure 5). Blom et al. (1997) found that a mixture of 2.5% SL and 0.25% acetate prevented the growth of *L. monocytogenes* in servelat sausage while maintaining the sensory acceptability of the sausage. Zhu et al. (2005) reported that 2% SL and 0.1% SD in combination with low-dose irradiation were effective in ensuring the safety of ready-to-eat meat products against *L. monocytogenes* SL increased the firmness and saltiness of turkey hams, but its overall impact on quality was minimal. Since the SL concentration used in our study was lower than 2% (w/w), we would assume that the chitosan-coated plastic film containing 10 mg/cm^2 of SL might not affect the sensory quality of ham steaks. Chitosan itself is a polymer that

imparts minimal adverse sensory properties to food. Jo et al. (2001) investigated the quality properties of pork sausage prepared with water-soluble chitosan oligomer. No difference in color, flavor, texture, overall acceptance, and mechanical texture was detected. The quality of the sausage with added chitosan oligomer (0.2%) was acceptable. As such, we would assume that SL and chitosan utilized at the levels described in our study would not be of an organoleptic concern.

The total aerobic and anaerobic counts in the un-inoculated samples during storage were also determined in this study as an index of microbial quality (Data not shown). Throughout the 12-week storage study, aerobic counts were ≤ 2.1 log CFU/cm^2 in plain film samples and ≤ 1.0 log CFU/cm^2 in the chitosan and chitosan with SL samples. The anaerobic counts in all the samples were ≤ 1.1 log CFU/cm^2 during the storage. These counts were considered low and the bacteria in the samples would thus not be expected to encourage or inhibit *L. monocytogenes*.

3.4. **Conclusion**

In this study, effect of chitosan-coated film on the growth of *L. monocytogenes* was first investigated in an aqueous system of TSBYE broth and chitosan-coated films were found to inhibit the growth in a concentration-dependent manner. LMW chitosan had a better antilisterial effect than MMW chitosan when used in an aqueous system. However, chitosan-coated plastic films did not inhibit the growth of *L. monocytogenes* on ham steaks. Incorporation of antimicrobials, nisin (500 IU/cm^2), SD (2.5 mg/cm^2), SL (10 mg/cm^2), SB (1 mg/cm^2), and PS (3 mg/cm^2), into

chitosan-coated films considerably enhanced their effectiveness against *L. monocytogenes*. Chitosan-coated film containing SL was the most effective among the five antimicrobial films studied and showed excellent long-term antilisterial effectiveness on ham steaks. Before commercial application of any of the effective treatment described in this study, sensory quality studies should be carried out.

References

Appendini, P. and Hotchkiss, J. H. (2002) Review of antimicrobial food packaging. *Innov. Food Sci. Emerg. Technol.* 3, 113-126.

Bedie, G. K., Samelis, J., Sofos, J. N., Belk, K. E., Scanga, J. A. and Smith, G. C. (2001) Antimicrobials in the formulation to control *Listeria monocytogenes* postprocessing contamination on frankfurters stored at 4 degrees C in vacuum packages. *J. Food Prot.* 64, 1949-1955.

Benkerroum, N. and Sandine, W. E. (1988) Inhibitory action of nisin against *Listeria monocytogenes*. *J. Dairy Sci.* 71, 3237-3245.

Besse, N. G., Audinet, N., Beaufort, A., Colin, P., Cornu, M. and Lombard, B. (2004) A contribution to the improvement of *Listeria monocytogenes* enumeration in cold-smoked salmon. *Int. J. Food Microbiol.* 91, 119-127.

Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E., Nissen, H. and Nesbakken, T. (1997) Addition of 2.5% lactate and 0.25% acetate controls growth of *Listeria monocytogenes* in vacuum-packed, sensory-acceptable servelat sausage and cooked ham stored at 4 degrees C. *Int. J. Food Microbiol.* 38, 71-76.

CDC. (1999) Update: Multistate outbreak of listeriosis—United States, 1998–1999. *Morb. Mortal. Wkly. Rep.* 47, 1117-1118.

CDC. (2000) Multistate outbreak of listeriosis—United States, 2000. *Morb. Mortal. Wkly. Rep.* 49, 1129-1130.

CDC. (2002) Public health dispatch: Outbreak of listeriosis—Northeastern United States, 2002. *Morb. Mortal. Wkly. Rep.* 51, 950-951.

Coma, V., Sebti, I., Pardon, P., Deschamps, A. and Pichavant, F. H. (2001) Antimicrobial edible packaging based on cellulosic ethers, fatty acids, and nisin incorporation to inhibit *Listeria innocua* and *Staphylococcus aureus. J. Food Prot.* 64, 470-475.

Delves-Broughton, J. and Gasson, M. J. (1994) Nisin. In Natural antimicrobial systems in food preservation (Eds Dillon, V.M. and Board, R.G.) pp. 99-132. Wallingford, UK. CAB International.

Doores, S. (1993) Organic acids. In Antimicrobials in foods, 2nd ed. (Eds P. M. Davidson and Branen, A. L.) pp. 95-136. New York. Marcel Dekker.

El-Shenawy, M. A. and Marth, E. H. (1988) Inhibition or inactivation of *Listeria monocytogenes* by sorbic acid. *J. Food Prot.* 51, 842-847.

Ghanem, A. and Skonberg, D. (2002) Effect of preparation method on the capture and release of biologically active molecules in chitosan gel beads. *J. Appl. Polym. Sci.* 84, 405-413.

Glass, K. A., Granberg, D. A., Smith, A. L., McNamara, A. M., Hardin, M., Mattias, J., Ladwig, K. and Johnson, E. A. (2002) Inhibition of *Listeria monocytogenes* by sodium diacetate and sodium lactate on wieners and cooked bratwurst. *J. Food Prot.* 65, 116-123.

Harris, L. J., Fleming, H. P. and Klaenhammer, T. R. (1991) Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A, and UAL500 to nisin. *J. Food Prot.* 54, 836-840.

Helander, I. M., Nurmiaho-Lassila, E., Ahvenainen, R., Rhoades, J. and Roller, S. (2001) Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Int. J. Food Microbiol.* 71, 235-244.

Hurst, A. and Hoover, D. G. (1993) Nisin. In Antimicrobials in foods (Eds Davidson P.M. and Branen, A.L.) pp. 369-407. New York. Marcel Dekker.

Islam, M., Chen, J., Doyle, M. P. and Chinnan, M. (2002) Control of *Listeria monocytogenes* on turkey frankfurters by generally-recognized-as-safe preservatives. *J. Food Prot.* 65, 1411-1416.

Janes, M. E., Kooshesh, S. and 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. *J. Food Sci.* 67, 2754-2757.

Jay, J. M. (2000) Modern Food Microbiology, 6th ed. Gaithersburg, MD. Aspen Publishers, Inc.

Jeon, Y., Park, P. and Kim, S. (2001) Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydr. Polym.* 44, 71-76.

Jo, C., Lee, J. W., Lee, K. H. and Byun, M. W. (2001) Quality properties of pork sausage prepared with water-soluble chitosan oligomer. *Meat Sci.* 59, 369-375.

Kang, D. H. and Fung, D. Y. C. (1999) Thin agar layer method for recovery of heat-injured *Listeria monocytogenes. J. Food Prot.* 62, 1346-1349.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z. and Yao, K. D. (2001) Antibacterial action of chitosan and carboxymethylated chitosan. *J. Appl. Polym. Sci.* 79, 1324-1335.

Lu, Z., Sebranek, J. G., Dickson, J. S., Mendonca, A. F. and Bailey, T. B. (2005) Inhibitory effects of organic acid salts for control of *Listeria monocytogenes* on frankfurters. *J. Food Prot.* 68, 499-506.

Lungu, B. and Johnson M. G. (2005) Fate of *Listeria monocytogenes* inoculated onto the surface of model turkey frankfurter pieces treated with zein coatings containing nisin, sodium diacetate, and sodium lactate at 4°C. *J. Food Prot.* 68, 855-859.

Mead, P. S., Slutsker, L., Dietz, V., McCraig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. and Tauxe, R. V. (1999) Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5, 607-625.

Moller, H., Grelier, S., Pardon, P. and Coma, V. (2004) Antimicrobial and physicochemical properties of chitosan-HPMC-based films. *J. Agric. and Food Chem.* 52, 6585-6591.

Neetoo, H., Ye, M. and Chen, H. (2007) The effectiveness and shelf-life of plastic films coated with nisin for inhibition of *Listeria monocytogenes*. *J. Food Prot.* 70, 1267-1271.

No, H. K., Park, N. Y. Lee, S. H. and Meyers, S. P. (2002) Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.* 74, 65-72.

Ouattara, B., Simard, R. E., Piette, G., Begin, A. and Holley, R. A. (2000) Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int. J. Food Microbiol.* 62, 139-148.

Prazak, A. M., Murano, E. A., Mercado, I. and Acuff, G. R. (2002) Antimicrobial resistance of *Listeria monocytogenes* isolated from various cabbage farms and packing sheds in Texas. *J. Food Prot.* 65, 1797-1799.

Quintavalla, S. and Vicini, L. (2002) Antimicrobial food packaging in meat industry. *Meat Sci.* 62, 373-380.

Ravi Kumar, M. N. V. (2000) A review of chitin and chitosan applications. *React. Funct. Polym.* 46, 1-27.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A. and Smith, G. C. (2002) Control of *Listeria monocytogenes* with combined antimicrobials after postprocess contamination and extended storage of frankfurters at 4 degrees C in vacuum packages. *J. Food Prot.* 65, 299-307.

Schlyter, J. H., Glass, K. A., Loeffelholz, J., Degnan, A. J. and Luchansky, J. B. (1993) The effects of diacetate with nitrite, lactate, or pediocin on the viability of *Listeria monocytogenes* in turkey slurries. *Int. J. Food Microbiol.* 19, 271-281.

Shahamat, M., Seaman, A. and Woodbine, M. (1980) Survival of *Listeria monocytogenes* in high salt concentrations. *Zbl. Bakteriol. Hyg. I. Abt. Orig. A.* 246, 506-511.

Shahidi, F., Arachchi, J. K. V. and Jeon, Y. J. (1999) Food applications of chitin and chitosans. *Trends Food Sci. Technol.* 10, 37-51.

Shelef, L. A. (1994) Antimicrobial effects of lactates: A review. *J. Food Prot.* 57, 445-450.

Sofos, J. N. and Busta, F. F. (1993). Sorbic acid and sorbates. In Antimicrobials in foods (Eds Davidson P.M. and Branen, A.L.) pp. 49-94. New York. Marcel Dekker.

Sudarshan, N. R., Hoover, D. G. and Knorr, D. (1992) Antibacterial action of chitosan. *Food Biotechnol.* 6, 257-272.

Szabo, E. A. and Cahill, M. E. (1999) Nisin and ALTA^{TM} 2341 inhibit the growth of *Listeria monocytogenes* on smoked salmon packaged under vacuum or 100% CO_2. *Lett. Appl. Microbiol.* 28, 373-377.

Thomas, L. V. and Delves-Broughton, J. (2005) Nisin. In Antimicrobials in food (Eds Davidson, P. M., Sofos, J. N. and Branen, A.L.) pp. 237-274. Boca Raton, FL. Taylor & Francis Group.

Tompkin, R. B. (2002) Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65, 709-725.

Ukuku, D. O. and Shelef, L. A. (1997) Sensitivity of six strains of *Listeria monocytogenes* to nisin. *J. Food Prot.* 60, 867-869.

USDA-FSIS. (2000) FSIS to increase permissible levels of food ingredients used as antimicrobials and flavoring agents. *Fed. Regist.* 65, 3121-3123.

USDA-FSIS. (2006) Isolation and identification of *Listeria monocytogenes* from red meat, poultry, egg, and environmental samples. Retrieved July 24, 2007, from http://www.fsis.usda.gov/PDF/MLG_8_05.pdf

Vojdani, F. and Torres, J. A. (1989) Potassium sorbate permeability of polysaccharide films: Chitosan, methylcellulose and hydroxypropyl methylcellulose. *J. Food Proc. Eng.* 12, 33-48.

Wakabayashi, H., Bellamy, W., Takase, M. and Tomita, M. (1992) Inactivation of *Listeria monocytogenes* by lactoferricin, a potent antimicrobial peptide derived from cow's milk. *J. Food Prot.* 55, 238-240.

Wang, G. H. (1992) Inhibition and inactivation of five species of foodborne pathogens by chitosan. *J. Food Prot.* 55, 916-919.

Zhu, M. J., Mendonca, A., Ismail, H. A., Du, M., Lee, E. J. and Ahn, D. U. (2005) Impact of antimicrobial ingredients and irradiation on the survival of *Listeria monocytogenes* and the quality of ready-to eat turkey ham. *Poult. Sci.* 84, 613-620.

Zivanovic, S., Basurto, C. C., Chi, S., Davidson, P. M. and Weiss, J. (2004) Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. *J. Food Prot.* 67, 952-959.

Figures

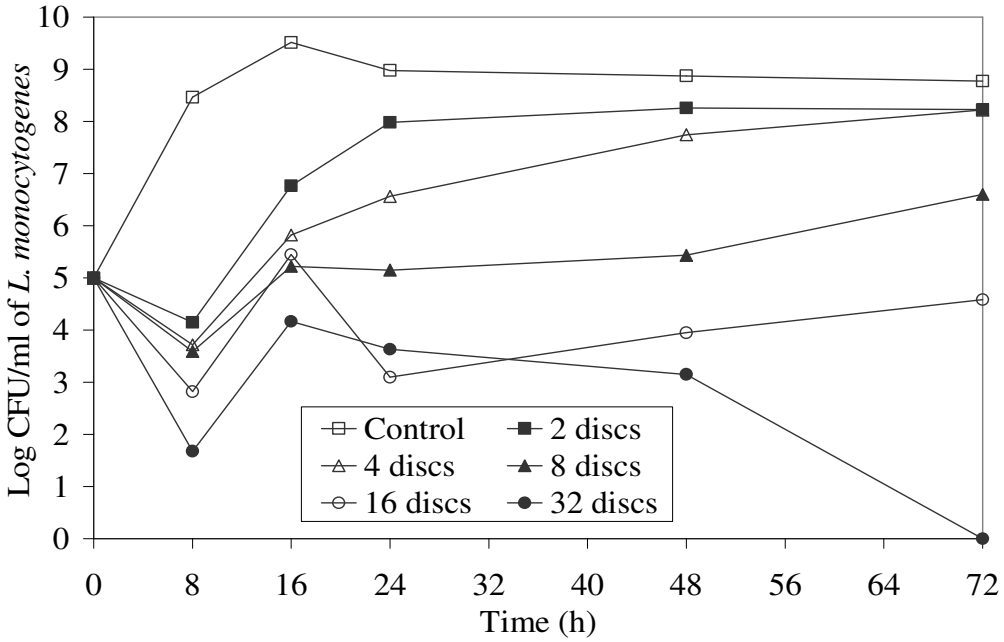

Fig. 3.1 Effect of chitosan-coated plastic film on the growth of *L. monocytogenes* in TSBYE broth.

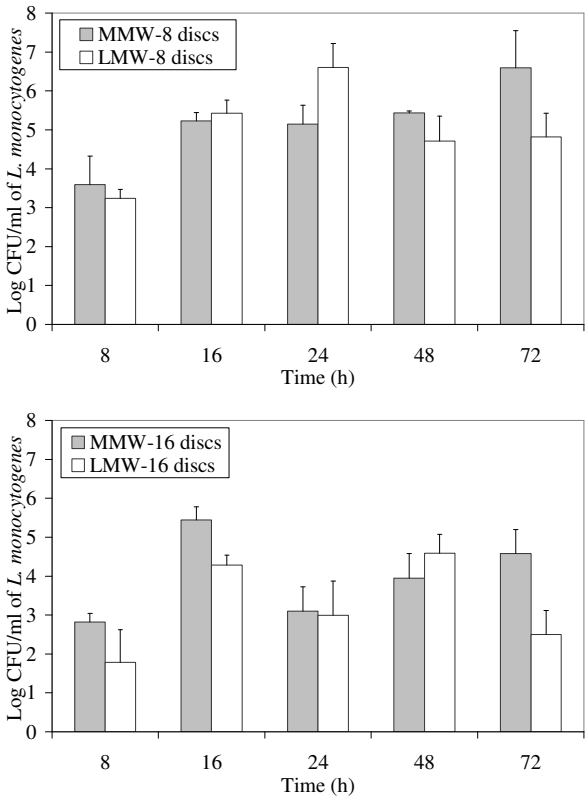

Fig. 3.2 Comparison of the antilisterial effectiveness of low and medium molecular weight chitosan-coated plastic films. Error bars represent ± 1 standard deviation.

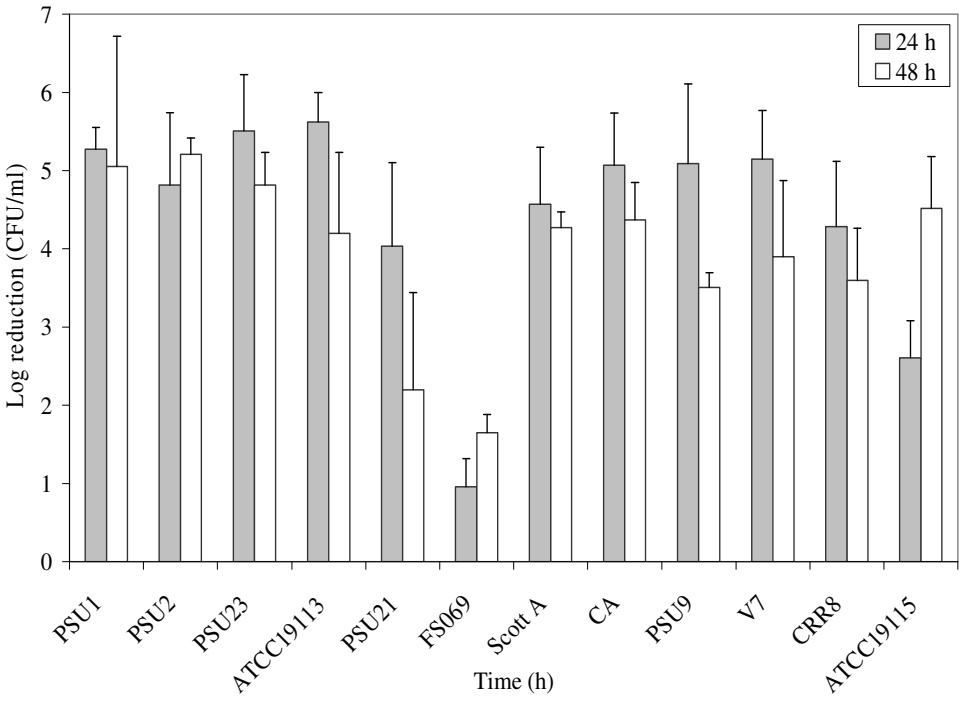

Fig. 3.3 Resistances of 12 strains of *L. monocytogenes* to low molecular weight chitosan-coated plastic film. Log reductions = log (counts in the control samples at 24 or 48 h for individual strains) – log (counts in the treated samples at 24 or 48 h for the correspondent strains). Error bars represent ± 1 standard deviation.

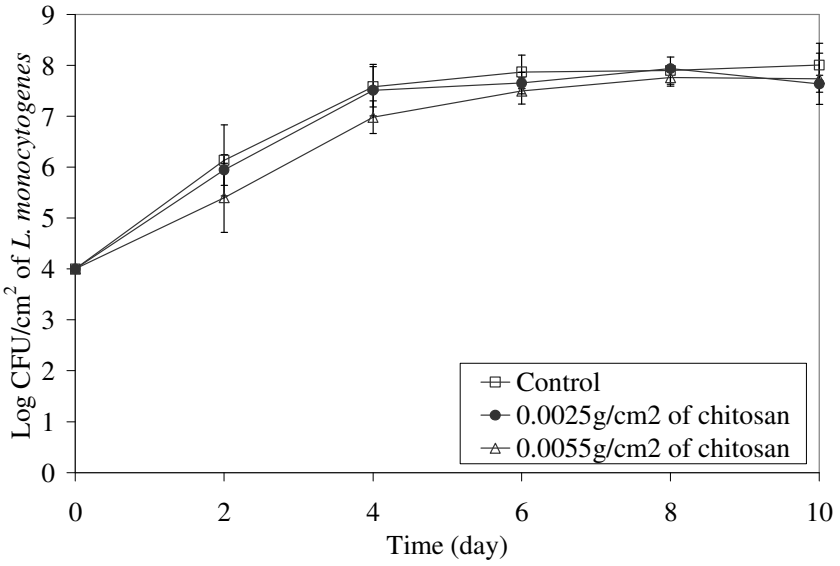

Fig. 3.4 Effect of chitosan-coated plastic films on the growth of *L. monocytogenes* on ham steaks stored at room temperature. Error bars represent ± 1 standard deviation.

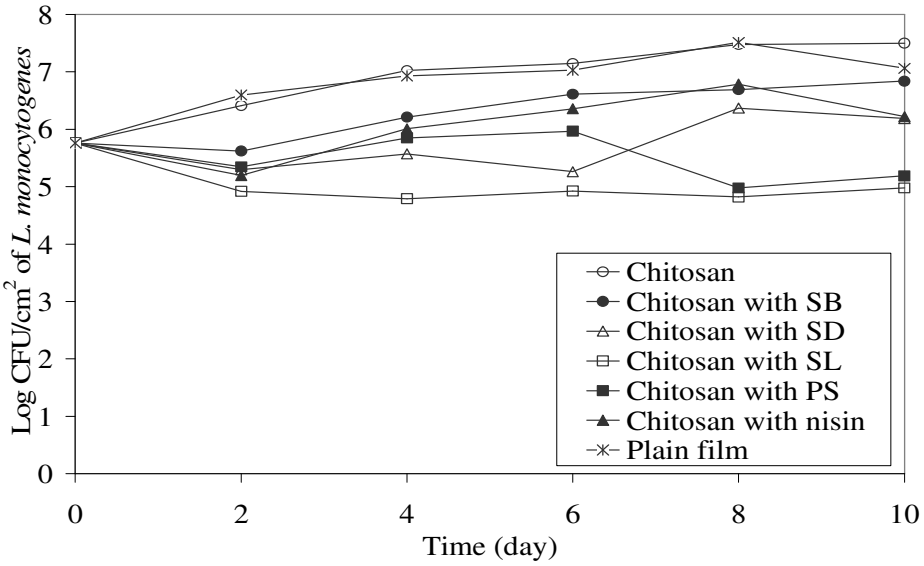

Fig. 3.5 Effect of chitosan-coated plastic films incorporating GRAS antimicrobials on the growth of *L. monocytogenes* on ham steaks stored at room temperature.

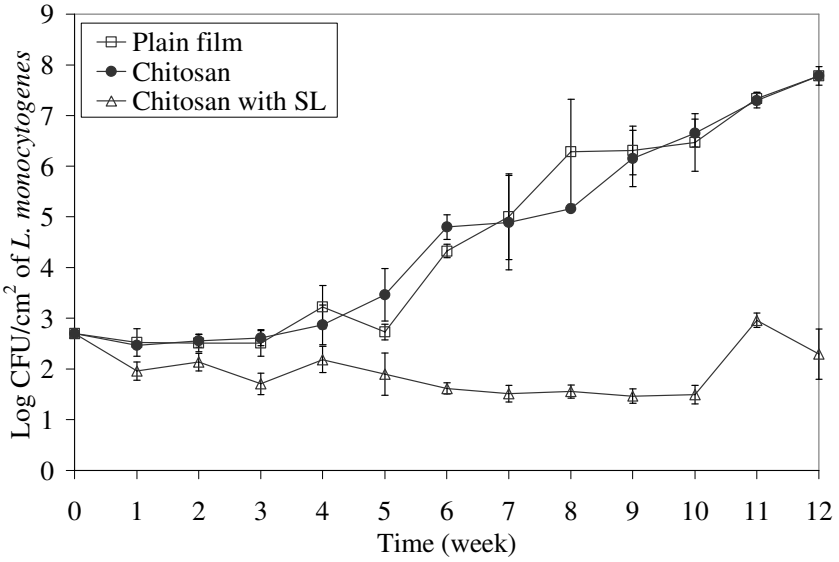

Fig. 3.6 Effect of chitosan-coated plastic films incorporating sodium lactate on the growth of *L. monocytogenes* on ham steaks stored at 4°C. Error bars represent ± 1 standard deviation.

Chapter 4

EFFECTIVENESS OF CHITOSAN-COATED PLASTIC FILMS INCORPORATING ANTIMICROBIALS IN INHIBITION OF *LISTERIA MONOCYTOGENES* ON COLD-SMOKED SALMON

Abstract

The objective of this study was to evaluate the efficacy of chitosan-coated plastic films incorporating five Generally Recognized as Safe (GRAS) antimicrobials (nisin, sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PS) and sodium benzoate (SB)) against *Listeria monocytogenes* on cold-smoked salmon. Salmon samples were surface-inoculated with a five-strain cocktail of *L. monocytogenes* and packaged in chitosan-coated plastic films containing 500 IU/cm^2 of nisin, 9 mg/cm^2 of SL, 0.5 mg/cm^2 of SD, 0.6 mg/cm^2 of PS, or 0.2 mg/cm^2 of SB, and stored at room temperature (ca. 20°C) for 10 days. The film incorporating SL was the most effective with counts of *L. monocytogenes* more than 3.2 log CFU/cm^2 lower than those of the controls after 10 days of storage. The other four antimicrobial films showed similar inhibition effects with counts more than 2.0 log CFU/cm^2 lower than those of the controls after 10 days of storage. The antilisterial efficacy of films containing lower

concentrations of SL (2.3 mg/cm^2 and 4.5 mg/cm^2) and binary combinations SL, PS, SD, SB and nisin were subsequently evaluated. Among all the treatments, chitosan-coated plastic films with 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin were the most effective. These three most effective antimicrobial films were then tested at refrigerated temperature. They completely inhibited the growth of *L. monocytogenes* on smoked salmon for at least 6 weeks. Chitosan-coated plastic films containing 4.5 mg/cm^2 SL can potentially assist the smoked salmon processing industry in their efforts to control *L. monocytogenes*.

4.1. Introduction

Listeria monocytogenes is a major safety concern for ready-to-eat (RTE) foods. This intracellular organism is a causative agent of listeriosis, a severe invasive illness in humans with a high fatality rate of approximately 30% (Vazquez-Boland et al., 2001). *L. monocytogenes* has been incriminated in several recent foodborne illness outbreaks associated with RTE foods (Centers for Disease Control and Prevention (CDC), 1999; 2000; 2002). As a facultatively anaerobic and psychrotrophic bacterium, *L. monocytogenes* can grow in vacuum-packaged and cold-stored RTE foods such as cold-smoked fish products. These foods generally have extended shelf-lives at refrigeration temperatures, are capable of supporting the growth of *L. monocytogenes*, and are eaten without cooking (Huss et al., 2000). The consumption of fish or seafood has been associated with human listeriosis outbreaks (Lennon et al., 1984; Ericsson et al., 1997; Brett et al., 1998; Jemmi et al., 2002). In a review of cold-smoked salmon

samples, Embarek (1994) reported a contamination rate of between 0% and 75%, with an overall prevalence of 10%. Heinitz and Johnson (1998) documented that 17.5% of cold-smoked fish and 8.1% of hot-smoked fish from the United States were contaminated with *L. monocytogenes.* Since cold-smoked salmon probably cannot be produced completely free of *L. monocytogenes* (Gram, 2001), intervention treatments are needed to prevent the growth of this pathogen and thus ensure safety of this product.

The use of antimicrobial packaging films can be a promising tool for controlling *L. monocytogenes* on RTE foods (Janes et al., 2002; Lungu and Johnson, 2005). Chitosan is a natural polymer which is nontoxic, biodegradable, and biocompatible. It is a good choice for antimicrobial films because of its superior film-forming properties, ability to adsorb nutrients used by bacteria, and capacity to bind water and inhibit various bacterial enzyme systems (Young et al., 1982; Darmadji and Izumimoto, 1994). Chitosan has intrinsic antimicrobial activity; however, chitosan film alone had no inhibitory effect on the growth of *L. monocytogenes* when applied to the surface of ham steaks (Ye et al., 2008). Therefore, in this study chitosan was coated onto a plastic film and used as a carrier for antimicrobials. Since edible film formed by chitosan is brittle and does not have good mechanical properties, coating chitosan onto a plastic film would overcome these problems. The additional benefit is that chitosan is not consumed with food. The chitosan-coated plastic films containing antimicrobials would allow the antimicrobials to be released gradually from the

chitosan coating and thus maintain a relatively high concentration of the antimicrobials on the food surface for a longer period of time.

In this study, the following five Generally Recognized as Safe (GRAS) antimicrobials were evaluated. Nisin exerts rapid inhibitory effects against gram-positive bacteria including *L. monocytogenes* in laboratory media or model food systems (Delves-Broughton and Gasson, 1994) and in RTE products including smoked salmon (Nilsson et al., 1997; Geornaras et al., 2006; Neetoo et al., 2008). Sodium lactate (SL) is used as a flavor enhancer in meat. SL has shown antilisterial effect in comminuted chicken, beef (Shelef and Yang, 1991), cook-in-bag roasts (Unda et al., 1991) and comminuted salmon model systems (Pelroy et al., 1994). Sodium diacetate (SD) is approved as a direct ingredient for use in foods. At 0.1 to 0.3%, SD can control growth of *L. monocytogenes* in meat (Schlyter et al., 1993; Ghanem and Skonberg, 2002). Both potassium sorbate (PS) and sodium benzoate (SB) have been shown to inhibit growth of *L. monocytogenes* in media, as well as in and on meat systems (El-Shenawy and Marth, 1988; Wederquist et al., 1994; Islam et al., 2002; Samelis et al., 2001). The maximum legal limits for use in foods in the U.S. are 10,000 IU/g (0.025%) for nisin in RTE foods (Code of Federal Regulations, 2003), 4.8% for SL in meats, 0.25% for SD in meats (Code of Federal Regulations, 2000), 0.1% for SB in foods (Code of Federal Regulations, 2007), and 0.3% for PS in meat and fish products (Sofos, 1989). The maximum limits allowed by the European Commission for these antimicrobials in foods are: nisin (0.00125% or 500 IU/g for ripened cheese and

processed cheese), PS (0.2%), SB (0.2%), while no upper limits are imposed for SL and SD (Nordic Council of Ministers, 2000).

The overall objective of this study was to develop effective antimicrobial films for controlling *monocytogenes* on vacuum-packaged cold-smoked salmon. The antilisterial efficacy of the chitosan-coated film containing the five individual GRAS antimicrobials was initially screened during storage at room temperature (20°C). Then single and binary combinations of these antimicrobials were evaluated during storage at room temperature. Finally, the most effective antimicrobial films were chosen and their antimicrobial efficacy with respect to *L .monocytogenes* and spoilage flobiota on cold-smoked salmon was assessed during refrigerated storage (4°C) for 8 weeks.

4.2. Materials and Methods

4.2.1. Screening for effective antimicrobials against *L. monocytogenes* on cold-smoked salmon

4.2.1.1. Preparation of antimicrobial films.

Two grams of low molecular weight (LMW) chitosan (Sigma-Aldrich, St. Louis, MO) were dissolved in 100 ml of 1% (w/v) acetic acid and stirred overnight at room temperature (chitosan concentration = 0.02 g/ml or 2%). Hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich) solution was prepared by dissolving 3 g of HPMC in 100 ml of 1% acetic acid. The coating solution was prepared by mixing 13 parts of chitosan solution with 2 parts of HPMC solution. Nisin (Sigma-Aldrich), SD (Sigma-Aldrich), SL (Fisher), SB (Fisher), and PS (Fisher) were separately

incorporated into the coating solution. The antimicrobial coating solutions were prepared by mixing 0.2 g of nisin, 0.2 g of SD, 6.0 g of a 60% SL syrup, 0.08 g of SB, or 0.24 g of PS with 15 ml of the coating solution. A 1.0-mil Surlyn® film (Printpack Inc., Atlanta, GA) was taped to 20 x 20 cm glass plates and the antimicrobial coating solution was cast onto the plastic film using a thin-layer chromatography plate coater (TLC, CAMAG, Muttenz, Switzerland). The gate of the TLC coater was fixed at 500 μm to control the thickness of the coating. The coated films contained 0.7 mg/cm^2 of chitosan incorporating 500 IU/cm^2 of nisin, 9 mg/cm^2 of SL, 0.5 mg/ cm^2 of SD, 0.6 mg/cm^2 of PS, or 0.2 mg/cm^2 of SB. A plain Surlyn® film and a film coated with 0.7 mg/cm^2 of chitosan without antimicrobials were used as controls. The coated films were air-dried at room temperature overnight.

4.2.1.2 Inoculation of cold-smoked salmon samples.

A cocktail of five strains of *L. monocytogenes* was used to increase genetic variability. These strains included PSU1 (Serotype 1/2a) (highly resistant to nisin), F5069 (Serotype 4b) (highly resistant to chitosan), and V7 (Serotype 1/2a) (highly resistant to organic salts), and two other strains, PSU9 (Serotype 1/2b) and Scott A (Serotype 4b). All strains were maintained on tryptic soy agar plus 0.6% yeast extract (TSAYE) (Difco Laboratories, Detroit, MI) agar plates at 4°C. The cultures were transferred monthly onto a freshly made TSAYE agar plate during the experimental period. For growth, a single colony of *L. monocytogenes* was inoculated into a tube of tryptic soy broth plus 0.6% yeast extract (TSBYE) (Difco) broth and

incubated at 35°C for 24 h. The culture was then transferred to 10 ml of fresh TSBYE and incubated for 24 h at 35°C to reach a final concentration of approximately 10^9 CFU/ml. A 1-ml volume of each culture was pooled to provide the cocktail.

Freshly processed cold-smoked salmon (*Salmo salar*) samples were obtained from a producer. They were kept frozen at -20°C and thawed at 2 ± 2°C (< 4°C) for 1 day immediately before use as described by Besse et al. (2004). Slices of smoked salmon were punched aseptically into 5.7-cm diameter round pieces weighing 10 ± 1 g with a surface area of 25.7 cm^2 on one side. The salmon discs were placed onto a piece of sterile aluminum foil, 125 µl of the five-strain cocktail was spread on one side of the salmon surface, and the samples were left undisturbed for 5 min to allow the inoculum to soak in and the cells to attach. Salmon discs were then flipped and the same procedure was repeated for the other side of each sample thus achieving final concentration of 5 x 10^5 CFU/cm^2 of salmon surface. After inoculation, salmon samples were kept at 4 °C for 20 min to allow bacterial attachment.

4.2.1.3. Packaging of inoculated cold-smoked salmon samples with antimicrobial films.

The inoculated samples were wrapped in the antimicrobial and control films prepared as described above. The wrapped samples were then inserted into 3-mm thick high barrier pouches (nylon/polyethylene, Koch Supplies, Kansas City, MO) and subsequently sealed using a vacuum-packaging machine (Model Ultravac 225 with digital control panel, Koch Equipment, Kansas City, MO). The packages were stored

at 20°C for 10 days. Instead of using 4°C, a typical refrigerated storage temperature for this kind of products, a relative high temperature was used to accelerate the selection of the most effective antimicrobials against *L. monocytogenes.*

4.2.1.4. Analysis of *L. monocytogenes* in the samples.

The samples were analyzed for *L. monocytogenes* every 2 days over the storage period. For determination of *L. monocytogenes* counts, a package was opened aseptically and the contents were transferred to a sterile stomacher bag and homogenized for 2 min with 40 ml of 0.1% peptone water. Ten-fold serial dilutions were made using 0.1% peptone water. Counts of *L. monocytogenes* were determined by an overlay method (Kang and Fung, 1999). Briefly, the serial dilutions were spread-plated onto solidified TSAYE agar plates and the plates were incubated at 35°C for 3 h. Approximately 7 ml of modified Oxford medium (Difco) at 45°C was overlaid on the TSAYE plates. The plates were incubated at 35°C for 48 h and small black colonies with black haloes on the plates were counted. Occasionally, colonies were confirmed to be *L. monocytogenes* using a BAX™ for Screening/*Listeria monocytogenes* PCR assay (Qualicon-DuPont, Wilmington, DE). The numbers of *L. monocytogenes* per cm^2 were calculated by dividing the total count of *L. monocytogenes* per disc by the total surface area (51.4 cm^2).

4.2.2. Screening for effective binary combinations of antimicrobials against *L. monocytogenes* on cold-smoked salmon

In the second phase of this study, binary combinations of antimicrobials were further evaluated on cold-smoked salmon. Since 9 mg/cm^2 SL was very effective against *L. monocytogenes*, lower concentrations of 2.3 mg/cm^2 and 4.5 mg/cm^2 SL were evaluated in the effort to minimize the cost and adverse changes on the sensory quality of the product. SL at low and high concentrations alone and in combinations with PS (0.6 mg/cm^2), SD (0.5 mg/cm^2), SB (0.2 mg/cm^2) and nisin (500 IU/cm^2) were screened for their ability to inhibit *L. monocytogenes* on cold-smoked salmon. In addition, binary combinations of PS, SD, SB and nisin were also tested. Antimicrobial films were prepared as described above. A plain Surlyn® film was used as control. The chitosan-coated film without antimicrobials was not used as a control in this experiment since it behaved similarly as the plain film in the previous experiment. The inoculated samples were wrapped in the antimicrobial and control films, inserted into the high barrier pouches and vacuum packaged. The packages were stored at 20°C for 10 days. The counts of *L. monocytogenes* in the salmon samples were determined using the overlay method on days 5 and 10.

4.2.3. Refrigerated storage study

Cold-smoked salmon samples were surface-inoculated with the five-strain cocktail of *L. monocytogenes* to a final concentration of approximately 5 x 10^2 CFU/cm^2. Three antimicrobial films, 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin and two control films, a plain Surlyn® film and a film coated with 0.7 mg/cm^2 chitosan, were used to wrap the inoculated salmon

samples. Samples were vacuum-packaged as described above. Samples were stored at 4°C for 8 weeks and counts of *L. monocytogenes* were analyzed weekly. Total aerobic and anaerobic counts in un-inoculated salmon samples were also determined using TSAYE and anaerobic agar (Difco Laboratories), respectively. Plates were incubated at 35°C for 2 days. Presence of *L. monocytogenes* in the un-inoculated salmon samples was determined by a primary enrichment in UVM broth (Difco Laboratories) and a secondary enrichment in Fraser broth (Difco Laboratories) according to the USDA Microbiology Laboratory Guidebook (USDA-FSIS, 2006) at the beginning of the experiment.

4.2.4. Statistical analysis

Three independent trials were conducted for each experiment. Single samples were serially diluted and plated in duplicate at each sampling time. Colony counts were converted to log CFU/cm^2 and means and standard deviations were calculated. Statistical analysis was conducted using Microsoft® Office Excel 2003. One-way analysis of variance (ANOVA) was used to compare significant differences between treatments ($P < 0.05$).

4.3. Results and Discussion

4.3.1. Effect of chitosan-coated plastic films incorporating single GRAS antimicrobials on the growth of *L. monocytogenes* on cold-smoked salmon.

Figure 1 shows the effect of chitosan-coated plastic films containing the five antimicrobials on the growth of *L. monocytogenes* on smoked salmon. To make

the figure easy to read, error bars are not shown in this figure. The average standard deviation for all the data points was 0.4 log CFU/cm^2. The initial concentration of *L. monocytogenes* on inoculated smoked salmon samples was 5.6 log CFU/cm^2. *L. monocytogenes* in two control films, plain and chitosan-coated films, grew to 7.6 log CFU/cm^2 after only 2 days of storage at room temperature, demonstrating the ability for the pathogen to grow rapidly on vacuum packaged cold-smoked salmon without antimicrobials at ambient temperature. The counts in the two controls reached 8.8 log CFU/cm^2 after 10 days. Incorporating antimicrobials into chitosan slowed down or inhibited the growth of *L. monocytogenes*. SL was the most effective antimicrobial reducing the initial counts from 5.6 to 5.4 log CFU/cm^2 after 10 days of storage. The count of *L. monocytogenes* in this treatment was $\geq$ 3.2 log CFU/cm^2 lower than those in the two controls after 10 days at room temperature and the difference was statistically significant ($P < 0.05$). Moreover, the SL treatment consistently had lowest counts of *L. monocytogenes* than other treatments at all time points. Other antimicrobials, SB, PS, nisin and SD, slowed down the growth of *L. monocytogenes* with counts $\geq$ 1.0 log lower than those of the control after 10 days of storage. These four antimicrobials had similar inhibition effect on *L. monocytogenes*.

Chitosan has antimicrobial activity, which is effectively expressed in aqueous systems (Sudarshan et al., 1992; Wang, 1992). However, its antimicrobial properties may become negligible when chitosan is in the form of insoluble films as demonstrated in this study (Fig. 1). The growth of *L. monocytogenes* in salmon

samples wrapped in the chitosan-coated film and plain film was very similar. It is possible that chitosan is ineffective in films because it is unable to diffuse through a rigid food matrix such as salmon. Compared with chitosan, the five antimicrobials used in this study are relatively small molecules which are able to migrate from the chitosan film to the smoked salmon samples. The antimicrobials had varying efficacy. These differences might be due to the intrinsic properties of the antimicrobials themselves, the concentrations used, the rate of diffusion of the antimicrobials and possible interactions with food components.

When the unit of mg of antimicrobials/cm^2 of film surface was converted to the unit of g of antimicrobials/g of salmon, the concentrations of SL (9 mg/cm^2), SD (0.5 mg/cm^2), PS (0.6 mg/cm^2), and SB (0.2 mg/cm^2) used in this study were at their maximum legal limits of 4.8%, 0.25%, 0.3% and 0.1% (w/w). Nisin has a legal limit of 10,000 IU/g. A much lower nisin level, 500 IU/cm^2 or 2500 IU/g was used in this study since nisin is relatively expensive and the cost of using high nisin levels would be prohibitive for the food industry. SL was the most effective antimicrobial against *L. monocytogenes*; however, the concentration used in this study, 9 mg/cm^2 (4.8%, w/w), was considered to be relatively high. A level of 2% is typically recommended for meat and poultry products (Shelef, 1994), although higher concentrations are now deemed more effective. SL at concentrations of 2% to 3% was effective as antilisterial compounds in meat (Mbandi and Shelef, 2001). Wederquist et al. (1994) reported that 2.0% SL significantly slowed down the growth of *L. monocytogenes* in refrigerated

turkey bologna. There is a concern that high levels of chemical preservatives required to inhibit the growth of *L. monocytogenes* can have a negative impact on sensory properties and may discourage customers from purchasing the product. Moreover, antimicrobials may have other restrictions such as costs and solubility. Therefore, use of lower concentrations of antimicrobials would provide more acceptable options for processors. Thus, lower concentrations of SL, 2.3 mg/cm^2 (1.2%, w/w) and 4.5 mg/cm^2 (2.4%, w/w), were used in the next phase of this study. The efficacy of most antimicrobials was dose dependent, and use of antimicrobials in combination may increase their overall antimicrobial action, lowering the necessary concentration of individual substances (Jay, 2000). Therefore, in the second phase of this study, binary combinations of antimicrobials were evaluated on cold-smoked salmon.

4.3.2. Effect of chitosan-coated plastic films incorporating single and binary combinations of antimicrobials on the growth of *L. monocytogenes* on cold-smoked salmon.

Counts of *L. monocytogenes* for inoculated smoked salmon slices treated by SL alone and binary combinations of the five antimicrobials are shown in Table 1. The initial concentration of *L. monocytogenes* on inoculated smoked salmon was 5.7 log CFU/cm^2. On day 5, the control had significantly higher count (8.4 log CFU/cm^2) than all the other treatments ($P < 0.05$). On day 10, *L. monocytogenes* in the control (plain film) reached 8.8 log CFU/cm^2 while incorporation of antimicrobials into the chitosan-coated films produced significantly lower counts than the plain film ($P <$

0.05). The three treatments, 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin, were the most effective with counts ≥ 1.9 log lower than those in the control. Therefore, these three treatments were selected to be used in the following refrigerated storage study. Although the treatments of 4.5 mg/cm^2 SL-500 IU/cm^2 nisin and 0.6 mg/cm^2 PS-0.2 mg/cm^2 SB were among the most effective treatments on day 5, these two treatments did not have lasting antimicrobial effect and were not evaluated in the following study.

Typically, combination treatments have a greater effect than individual treatments at the same level (Bell and Kyriakides, 1998; Harmayani et al., 1993). Thus, combination of antimicrobials could potentially reduce the concentrations of each chemical used in food products. Mbandi and Shelef (2002) reported that combination of 1.8% SL and 0.1% SD was listeriostatic during storage for 30 days and was 1.3 times higher than the sum of reductions obtained with the single salts at 5°C. In a study conducted by Nykänen et al. (2000), the combination of nisin and lactate functioned very well in inhibiting the growth of *Listeria* in cold-smoked fish. The synergistic effect was clearly observed, since the combination contained half the amount of both additives (nisin or lactate) was more effective than either one in inhibiting the growth of *L. monocytogenes* and even decreased the level of *L. monocytogenes*.

4.3.3. Evaluation of long-term antilisterial effectiveness of selected antimicrobial combinations on cold- smoked salmon.

The three most effective treatments, 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin, were evaluated. Representative un-inoculated samples of cold-smoked salmon had no detectable *Listeria* spp.; therefore all *Listeria* spp. found in the inoculated samples originated from the inoculum and was *L. monocytogenes*. The mean population of *L. monocytogenes* on inoculated treated sample as recovered just after inoculation was 2.7 log CFU/cm^2. Counts of *L. monocytogenes* on salmon samples treated with chitosan-coated films incorporating selected chemicals are shown in Figure 2. The typical shelf life of cold-smoked salmon at refrigeration is about 6 weeks. An eight-week storage study was conducted to determine whether use of antimicrobial films could extend the microbiological shelf-life of smoked salmon. *L. monocytogenes* in the two controls, plain film and film coated with chitosan, grew rapidly and reached > 7.0 log CFU/cm^2 after 5 weeks storage at 4°C. The three antimicrobial films reduced the counts from 2.7 to ≤ 2.1 log CFU/cm^2 after the fist week and consistently inhibited the growth of *L. monocytogenes* for at least 6 weeks. After 6 weeks of storage, the populations of *L. monocytogenes* in samples wrapped with the three antimicrobial films were still lower than the initial inoculation level. The treatments of 4.5 g/cm^2 SL-0.6 mg/cm^2 PS and 4.5 mg/cm^2 SL were the most effective and completely inhibited the growth of *L. monocytogenes* in smoked salmon during 8 weeks of storage at 4°C. For the 2.3 mg/cm^2 SL-500 IU/cm^2 nisin treatment, *L. monocytogenes* started to grow slowly after 6 weeks and the population reached 3.4 log CFU/cm^2 after 8 weeks. Overall, chitosan-coated films

containing 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS was the most effective, followed by 4.5 mg/cm^2 SL and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin although there was no statistical difference among them ($P > 0.05$).

These results demonstrated that the effectiveness of antimicrobials films could be tested at room temperature to considerably decrease the testing time since the results obtained from this part of the study were in agreement with those obtained in the screening studies. The three most effective antimicrobial treatments selected from the second screening study were also effective at refrigerated temperature for long term storage. The results for chitosan-coated film without antimicrobials obtained from this part of the study were also in agreement with those obtained in the first screening study, no inhibition effect was observed. Since RTE foods typically have a relatively long shelf-life, testing the efficacy of antimicrobials in RTE foods at refrigerated temperature is time-consuming. The approach used in this study, testing at room temperature instead of refrigerated temperature, might provide a means for rapid screening of the efficacy of antimicrobials.

Our results for the refrigerated study are in agreement with those reported by other researchers. Pelroy et al. (1994) reported that 2% SL in combination with 3% sodium chloride inhibited growth of *L. monocytogenes* for up to 50 days in cold-smoked salmon at 5°C. Nykänen et al. (2000) found that both nisin and lactate inhibited the growth of *L. monocytogenes* in smoked fish, but the combination of the two compounds was even more effective. The combination of 3.6 % SL and 240–360

IU/g nisin or 1.8 % SL and 120–180 IU/g nisin injected into smoked fish kept the level of *L. monocytogenes* remaining almost constant (4.7 to 4.9 log CFU/g) for 29 d at 3°C in vacuum-packed cold-smoked rainbow trout samples. The treatments did not affect the sensory characteristics of cold-smoked rainbow trout. Vogel et al. (2006) injected brine supplemented with combinations of potassium lactate (PL) and sodium acetate (SA) or PL and SD into cold-smoked salmon and found that 2.1% PL and 0.12% SD delayed the growth of *L. monocytogenes* for up to 42 days of vacuum-packaged storage at 10°C. The preservatives in the brine-injected cold-smoked salmon did not adversely affect flavor or odor and did not introduce off-flavors. These results indicate that the chitosan-coated plastic film containing 4.5 mg/cm^2 SL (2.4% w/w) developed in this study might not affect the sensory of smoked salmon.

The total aerobic and anaerobic counts in the un-inoculated samples during storage were also determined in this study as an index of microbial quality. On day 0, the initial populations of aerobes and anaerobes in un-inoculated control samples were < 0 log CFU/cm^2. Throughout the 8-week storage study, the growth of aerobes was significantly retarded. The aerobic counts in the treated samples were significantly lower than those in the controls from the second week to the end of the study ($P < 0.05$) (Fig 3A). At the end of the 8-week storage, the aerobic counts were ≥ 6.4 log CFU/cm^2 in the two controls and ≤ 2.5 log CFU/cm^2 in the three antimicrobial treatments. The anaerobic counts in the three antimicrobial treatments maintained at very low levels (≤ 1.9 log CFU/cm^2) throughout the 8-week storage study, while the

counts in the two controls varied considerably from < 1 log CFU/cm^2 to a maximum level of 5.1 log CFU/cm^2. These results demonstrated that the antimicrobial films developed in this study could potentially be used to extend the microbial shelf-life of smoked salmon. Previous studies also reported the bacteriostatic effect of SL (Maca et al., 1999; Tan and Shelef, 2002). Wang (2000) found that 3% SL inhibited microbial as well as chemical changes of vacuum packaged Chinese-style sausage stored at 20°C and resulted in a shelf life extension of 25 days.

In summary, our results confirm that *L. monocytogenes* can grow to high levels on cold-smoked salmon, even at normal refrigeration temperature and especially at abusive temperature, if an intervention treatment is not applied. Chitosan-coated films containing 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL-500 IU/cm^2 nisin were the most effective treatments against *L. monocytogenes* at ambient temperature in this study and showed long-term antilisterial efficacy during refrigerated storage on vacuum packaged cold-smoked salmon. Since the chitosan-coated film containing, 4.5 mg/cm^2 SL was able to inhibit the growth of *L. monocytogenes* for 8 weeks at refrigerated temperature and also contained fewer antimicrobials than the one with 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS, it can potentially be uilized as an antimicrobial film to enhance the safety of smoked salmon by the smoked fish industry. It is hoped that data from this study would provide processors with viable formulation options for designing antimicrobial packages to improve the microbiological safety and quality of smoked salmon during its refrigerated shelf-life.

It should be pointed out though that formal sensory analysis need to be conducted before commercial application of any of the effective treatments described in this study.

References

Bell, C. and Kyriakides, A. (1998) *Listeria*: A practical approach to the organism and its control in foods. London, U.K. Blackie Academic & Professional.

Besse, N. G., Audinet, N., Beaufort, A., Colin, P., Cornu, M. and Lombard, B. (2004) A contribution to the improvement of *Listeria monocytogenes* enumeration in cold-smoked salmon. *Int. J. Food Microbiol.* 91, 119-127.

Brett, M. S. Y., Short, P.and McLauchlin, J. (1998) A small outbreak of listeriosis associated with smoked mussels. *Int. J. Food Microbiol.* 43, 223-229.

CDC. (1999) Update: Multistate outbreak of listeriosis—United States, 1998–1999. *Morb. Mort. Wkly. Rep.* 47, 1117-1118.

CDC. (2000) Multistate outbreak of listeriosis—United States, 2000. *Morb. Mort. Wkly. Rep.* 49, 1129-1130.

CDC. (2002) Public health dispatch: Outbreak of listeriosis—Northeastern United States, 2002. *Morb. Mort. Wkly. Rep.* 51, 950-951.

Code of Federal Regulations, U.S. FDA. (2000) Available at

http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/99-028DF.htm

Code of Federal Regulations, U.S. FDA. (2003) Available at

http://frwebgate6.access.gpo.gov/cgibin/waisgate.cgi?WAISdocID=789888418736+6+0+0&WAISaction=retrieve

Code of Federal Regulations, U.S. FDA. (2007) Available at

http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=184.1733

Darmadji, P. and Izumimoto, M. (1994) Effect of chitosan in meat preservation. *Meat Sci.* 38, 243-254.

Delves-Broughton, J. and Gasson, M. J. (1994) Nisin. In Natural antimicrobial systems in food preservation (Eds Dillon, V.M. and Board, R.G.) pp. 99-132. Wallingford, UK. CAB International.

El-Shenawy, M. A. and E. H Marth. (1988) Inhibition or inactivation of *Listeria monocytogenes* by sorbic acid. *J. Food Prot.* 51, 842-847.

Embarek, P. K. B. (1994) Presence, detection and growth of *Listeria monocytogenes* in seafoods: A review. *Int. J. Food Microbiol.* 23, 17-34.

Ericsson, H., Eklow, A., Danielsson-Tham, M., Loncarevic, S., Mentzing, L., Persson, I., et al. (1997). An outbreak of listeriosis suspected to have been caused by rainbow trout. *J. Clin. Microbiol.* 35, 2904-2907.

Georgnaras, I., Skandamis, P. N., Belk, K. E., Scanga, J. A., Kendall, P. A. and Smith, G. C. (2006) Postprocess control of *Listeria monocytogenes* on commercial frankfurters formulated with and without antimicrobials and stored at 10°C. *J. Food Prot.* 69, 53-61.

Ghanem, A. and Skonberg, D. (2002) Effect of preparation method on the capture and release of biologically active molecules in chitosan gel beads. *J. Appl. Polym. Sci.* 84, 405-413.

Gram, L. (2001) Potential hazards in cold-smoked fish: *Listeria monocytogenes*. *J. Food Sci.* 66, s1072-s1081.

Harmayani, E., Sofos, J. N. and Schmidt, G. R. (1993) Fate of *Listeria monocytogenes* in raw and cooked ground beef with meat processing additives. *Int. J. Food Microbiol.* 18, 223-232.

Heinitz, M. L. and Johnson, J. M. (1998) The incidence of *Listeria* spp., *Salmonella* spp., and *Clostridium botulinum* in smoked fish and shellfish. *J. Food Prot.* 61, 318-323.

Huss, H. H., Jorgensen, L. V. and Vogel, B. F. (2000). Control options for *Listeria monocytogenes* in seafoods. *Int. J. Food Microbiol.* 62, 267-274.

Islam, M., Chen, J., Doyle, M. P. and Chinnan, M. (2002) Control of *Listeria monocytogenes* on turkey frankfurters by generally-recognized-as-safe preservatives. *J. Food Prot.* 65, 1411-1416.

Janes, M. E., Kooshesh, S. and 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. *J. Food Sci.* 67, 2754-2757.

Jay, J. M. (2000) Modern Food Microbiology, 6th ed. Gaithersburg, MD. Aspen Publishers, Inc.

Jemmi, T., Pak, S. I. and Salman, M. D. (2002) Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland, 1992-2000. *Prev.Vet. Med.* 54, 25-36.

Kang, D. H. and Fung, D. Y. C. (1999) Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. *J. Food Prot.* 62, 1346-1349.

Lennon, D., Lewis, B., Mantell, C., Becroft, D. D., Dove, B., Farmer, K., Tonkin, S., Yeates, N., Stamp, R. and Micleson, K. (1984) Epidemic perinatal listeriosis. *Pediatr. Infect. Dis. J.* 312, 30-34.

Lungu, B. and Johnson M. G. (2005) Fate of *Listeria monocytogenes* inoculated onto the surface of model turkey frankfurter pieces treated with zein coatings containing nisin, sodium diacetate, and sodium lactate at 4°C. *J. Food Prot.* 68, 855-859.

Maca, J. V., Miller, R. K., Bigner, M. E., Lucia, L. M., and Acuff, G. R. (1999) Sodium lactate and storage temperature effects on shelf life of vacuum packaged beef top rounds. *Meat sci.* 53, 23-29.

Mbandi, E. and Shelef, L. A. (2001) Enhanced inhibition of *Listeria monocytogenes* and *Salmonella enteritidis* in meat by combinations of sodium lactate and diacetate. *J. Food Prot.* 64, 640-644.

Mbandi, E.and Shelef, L. A. (2002) Enhanced antimicrobial effects of combination of lactate and diacetate on *Listeria monocytogenes* and *Salmonella* spp. in beef bologna. *Int. J. Food Microbiol.* 76, 191-198.

Neetoo, H, Ye, M. Chen, H., Joerger, R.D., Hicks, D.T. and Hoover. D.G. (2008) Use of nisin-coated plastic films to control *Listeria monocytogenes* on vacuum-packaged cold-smoked salmon. *Int. J. Food Microbiol.* 122, 8-15.

Nilsson, L., Henrik Huss, H. and Gram, L. (1997) Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. *Int. J. Food Microbiol.* 38, 217-227.

Nordic Council of Ministers. 2002. Food Additives in Europe, 2000. Available at www.norfad.dk/download/NorFAD.pdf

Nykänen, A., Weckman, K. and Lapveteläinen, A. (2000) Synergistic inhibition of *Listeria monocytogenes* on cold-smoked rainbow trout by nisin and sodium lactate. *Int. J. Food Microbiol.* 61, 63-72.

Pelroy, G. A., Peterson, M. E., Holland, P. J. and Eklund, M. W. (1994) Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium lactate. *J. Food Prot.* 57, 108-113.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A. and Smith, G. C. (2005) Combinations of nisin with organic acids or salts to control *Listeria*

monocytogenes on sliced pork bologna stored at 4°C in vacuum packages. *LWT.* 38, 21-28.

Schlyter, J. H., Degnan, A. J., Loeffelholz, J., Glass, K. A. and Luchansky, J. B. (1993) Evaluation of sodium diacetate and ALTA 2341 on viability of *Listeria monocytogenes* in turkey slurries. *J. Food Prot.* 56, 808-810.

Shelef, L. A., 1994. Antimicrobial effects of lactates: a review. Journal of food protection 57, 445-450.

Shelef, L. A. and Yang, Q. (1991) Growth suppression of *Listeria monocytogenes* by lactates in broth, chicken, and beef. *J. Food Prot*. 54, 283-287.

Sofos, J. N. (1989) Sorbate Food Preservatives.CRC Press, pp. 132.

Sudarshan, N. R., Hoover, D. G. and Knorr, D. (1992) Antibacterial action of chitosan. *Food Biotechnol.* 6, 257-272.

Tan, W. and Shelef, L. A. (2002) Effects of sodium chloride and lactates on chemical and microbiological changes in refrigerated and frozen fresh ground pork. *Meat sci.* 62, 27-32.

Unda, J. R., Molins, R. A. and Walker, H. W. (1991) *Clostridium sporogenes* and *Listeria monocytogenes*: Survival and inhibition in microwave-ready beef roasts containing selected antimicrobials. *J. Food Sci.* 56, 198-205.

Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., et al. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Revi.* 14, 584-640.

Vogel, B. F., Ng, Y. Y., Hyldig, G., Mohr, M. and Gram, L. (2006) Potassium lactate combined with sodium diacetate can inhibit growth of *Listeria monocytogenes* in vacuum-packed cold-smoked salmon and has no adverse sensory effects. *J. Food Prot.* 69, 2134-2142.

Wang, F. S. (2000) Effects of three preservative agents on the shelf life of vacuum packaged Chinese-style sausage stored at 20 degrees C. *Meat sci.* 56, 67-71.

Wang, G. H. (1992) Inhibition and inactivation of five species of foodborne pathogens by chitosan. *J. Food Prot.* 55, 916-919.

Wederquist, H. J., Sofos, J. N. and Schmidt, G. R. (1994) *Listeria monocytogenes* inhibition in refrigerated vacuum packaged turkey bologna by chemical additives. *J. Food Sci.* 59, 498-500.

Ye, M., Neetoo, H. and Chen, H. (2007) Control of *Listeria monocytogenes* on ham steaks by antimicrobials incorporated into chitosan-coated plastic films. *Food Micro.* 25, 260–268.

Young, D. H., Kohle, H. and Kauss, H. (1982) Effect of chitosan on membrane permeability of suspension-cultured glycine max and phaseolus vulgaris cells. *Plant Physiol*. 70, 1449-1454.

Tables

Table 4.1. Inhibitory effect of chitosan-coated films incorporating single and binary combinations of antimicrobials against *L. monocytogenes* on cold-smoked salmon at ambient temperature.

Treatment	*Numbers of* L. monocytogenes[a] *(log CFU/cm^2)*	
	5 days	10 days
Plain film	8.4 ± 0.3 A [b]	8.8 ± 0.3 A
2.3 mg/cm^2 SL	7.4 ± 0.2 B	7.6 ± 0.0 B
4.5 mg/cm^2 SL	7.0 ± 0.1 B	6.7 ± 0.2 B
2.3 mg/cm^2 SL & 0.6 mg/cm^2 PS	7.1 ± 0.2 B	7.5 ± 0.2 B
4.5 mg/cm^2 SL & 0.6 mg/cm^2 PS	6.7 ± 0.6 B	6.9 ± 0.9 B
2.3 mg/cm^2 SL & 0.2 mg/cm^2 SB	7.2 ± 0.2 B	7.6 ± 0.6 B
4.5 mg/cm^2 SL & 0.2 mg/cm^2 SB	7.1 ± 0.5 B	7.3 ± 0.3 B
2.3 mg/cm^2 SL & 0.5 mg/cm^2 SD	7.6 ± 0.3 B	7.6 ± 0.5 B
4.5 mg/cm^2 SL & 0.5 mg/cm^2 SD	6.8 ± 0.4 B	7.2 ± 0.6 B
2.3 mg/cm^2 SL & 500 IU/cm^2 nisin	7.3 ± 0.3 B	6.9 ± 0.5 B
4.5 mg/cm^2 SL & 500 IU/cm^2 nisin	6.5 ± 0.8 B	7.2 ± 0.3 B
0.6 mg/cm^2 PS & 0.5 mg/cm^2 SD	6.6 ± 0.8 B	7.3 ± 0.4 B
0.6 mg/cm^2 PS & 0.2 mg/cm^2 SB	7.4 ± 0.2 B	7.5 ± 0.1 B
0.6 mg/cm^2 PS & 500 IU/cm^2 nisin	7.5 ± 0.3 B	7.3 ± 0.2 B
0.5 mg/cm^2 SD & 0.2 mg/cm^2 SB	7.0 ± 0.8 B	7.7 ± 0.3 B
0.5 mg/cm^2 SD & 500 IU/cm^2 nisin	7.6 ± 0.1 B	7.6 ± 0.1 B
0.2 mg/cm^2 SB & 500 IU/cm^2 nisin	7.5 ± 0.4 B	7.6 ± 0.1 B

[a] Initial concentration of *L. monocytogenes* was 5.7 ± 0.1 log CFU/cm^2.

[b] Numbers reported are log CFU/cm^2 at the time of sampling (mean ± Standard deviation; n=3); within each section of table, means bering the same letters in the same column are not significantly different (P>0.05).

Figures

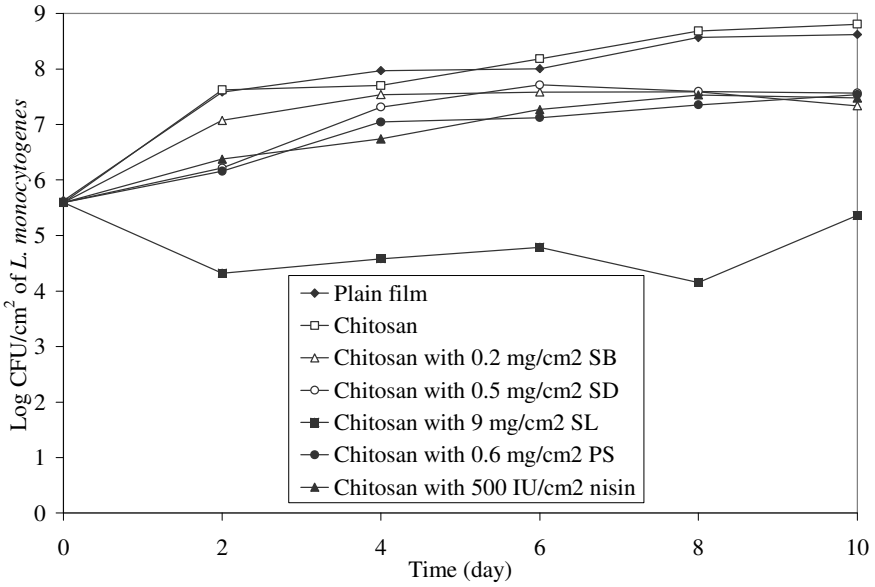

Fig. 4.1. Effect of chitosan-coated plastic films incorporating GRAS antimicrobials on the growth of *L. monocytogenes* on cold-smoked salmon stored at room temperature.

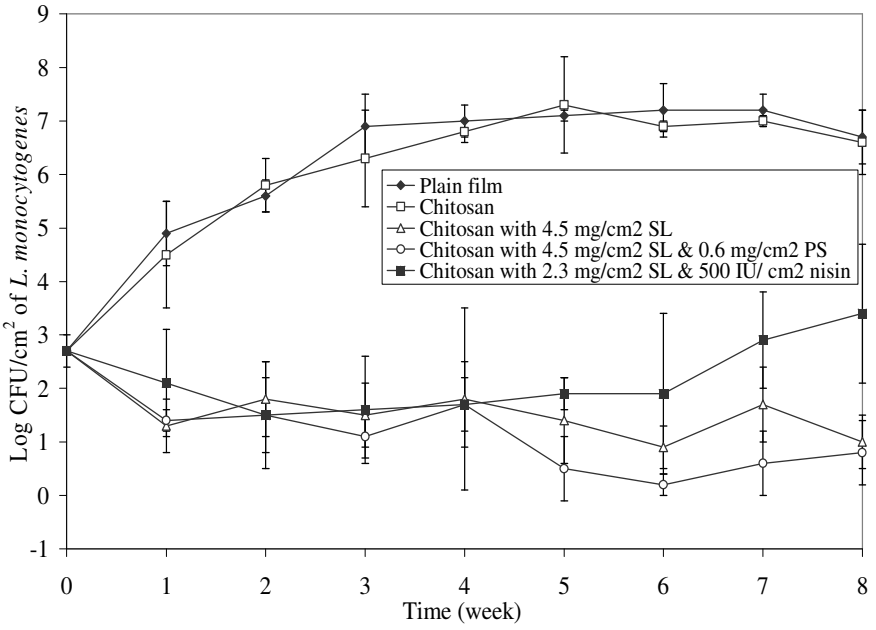

Fig. 4.2. Effect of chitosan-coated plastic films incorporating 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL- 500 IU/ cm^2 nisin on the growth of *L. monocytogenes* on cold-smoked salmon stored at 4°C. Error bars represent ± 1 standard deviation.

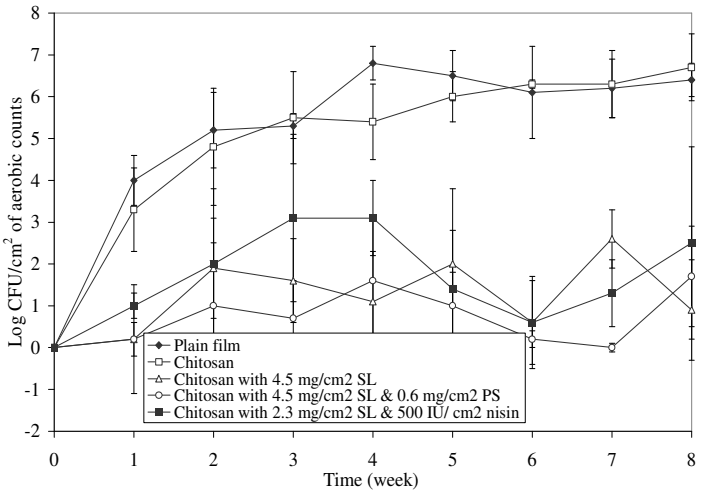

Fig. 4.3A

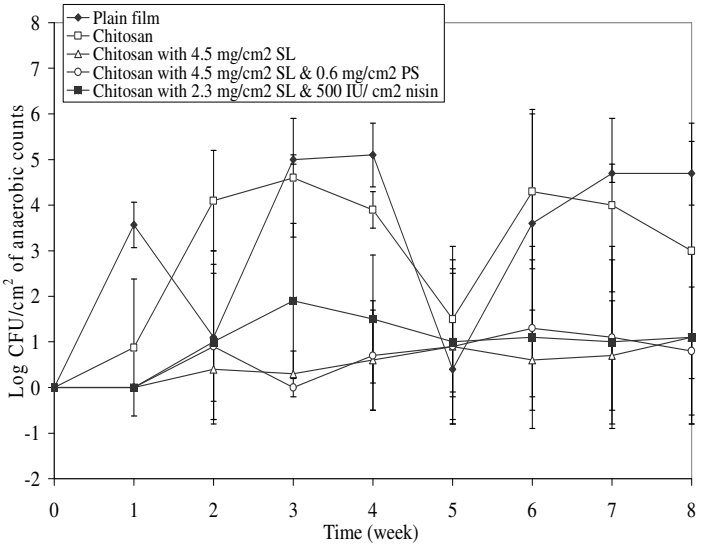

Fig. 4.3B

Fig. 4.3. Effect of chitosan-coated plastic films incorporating 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL-0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL- 500 IU/ cm^2 nisin on the growth of aerobes (Fig. 3A) and anaerobes (Fig. 3B) on cold-smoked salmon stored at 4°C. Error bars represent ± 1 standard deviation.

Chapter 5

CONCLUSION

In this project, effect of chitosan-coated film on the growth of *L. monocytogenes* was first investigated in an aqueous system of TSBYE broth and chitosan-coated films were found to inhibit the growth in a concentration-dependent manner. However, chitosan-coated plastic films did not inhibit the growth of *L. monocytogenes* on ham steaks. Incorporation of antimicrobials, nisin, SD, SL, SB, and PS, into chitosan-coated films considerably enhanced their effectiveness against *L. monocytogenes*. The chitosan-coated plastic film containing 10 mg/cm^2 of SL was the most effective antimicrobial film on ham steaks and showed excellent long-term antilisterial effect during 12-week storage at 4°C. Chitosan-coated plastic films with 4.5 mg/cm^2 of SL showed complete inhibition of *L. monocytogenes* on smoked salmon during 8-week storage at 4°C. Therefore, chitosan-coated plastic films containing SL could be used to control *L. monocytogenes* on ham steaks and cold-smoked salmon. Data from this study would provide processors with viable formulation options for antimicrobial packaging treatments to inhibit the growth of *L. monocytogenes* and spoilage bacteria during the refrigerated shelf-life.

Chapter 6

FUTURE RESEARCH

In summary, the application of antimicrobial films studied in this project on the surface of RTE food products can control the proliferation of *L. monocytogenes.* Before commercial application of any of the effective treatments described in this study, evaluations of the shelf life, chemical quality and sensory attributes of these food products need to be carried out. In addition, other agents such as antioxidants and flavor compounds could possibly be incorporated into chitosan films to improve the quality of RTE foods.

Inherent antimicrobial properties and film forming ability of chitosan make it ideal for use as biodegradable antimicrobial packaging material. However, the use of chitosan films has been restricted due to their inherent water sensitivity and relatively low stiffness and strength, especially in moist environments. If stand alone chitosan film (instead of coating chitosan onto a plastic film as described in this research) is preferred, more research is needed to develop antimicrobial chitosan films that are less sensitive to humidity. In addition, effects of chitosan production protocols, film-casting solvents, and plasticizer contents on sorption behavior could be further investigated to improve the functional properties of chitosan films. Moreover, for

application in food industry, use of less expensive chitosan-based antimicrobial films prepared from simplified production processes may be demanded.

Future investigation may include incorporation of antimicrobials (SL, SB, PS, nisin and other bacteriocins, lysozyme, essential oils, spices, etc.) into edible coatings to control the growth of *L. monocytogenes* and to extend shelf life of RTE foods. Edible coatings are applied directly and form a thin film on the food product, whereas films are self-supporting structures which are applied to the product after being formed separately. By incorporating antimicrobials, the functionality of edible coatings can be expanded to protect food products from microbial spoilage and extend shelf-life. Unlike a packaging film, an edible coating has an intimate and continuous association with the food until consumption. Incorporation of antimicrobials into edible coating or film to extend shelf life of fresh-cut fruits and vegetables is also of great merit for future research.