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ELIMINATING LISTERIA MONOCYTOGENES IN PACKED READY-TO-EAT POULTRY PRODUCTS BY COMBINING IN-PACKAGE PASTEURIZATION WITH NISIN AND/OR LYSOZYME

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ELIMINATING *LISTERIA MONOCYTOGENES* IN PACKED READY-TO-EAT POULTRY PRODUCTS BY COMBINING IN-PACKAGE PASTEURIZATION WITH NISIN AND/OR LYSOZYME

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ABSTRACT

Listeria monocytogenes is a significant food-borne pathogen particularly associated with ready-to-eat (RTE) meat and poultry products which can grow and multiply at refrigeration temperatures. In-package pasteurization is an effective post-lethality intervention to reduce microbial contamination of RTE meat products. Enhancing the efficiency of in-package pasteurization is vital in reducing the *L. monocytogenes* population as well as in preserving the quality of the meat product.

The first section of the dissertation research evaluated the effect of product thickness on the surface heating rate and final surface temperature during in-package pasteurization of vacuum-packaged bologna. Three thicknesses (4, 12, and 20 mm), corresponding to 1, 3, and 5 slices of two types of bologna having different (13 and 18%) fat contents were subjected to in-package pasteurization at 4 temperatures (60, 70, 80, and 90°C). Surface heating rate was fastest in the thinnest (4 mm) and slowest in the thickest (20mm) samples for all 4 temperatures. Final surface temperature attained after 3 min was lower with increased thickness levels for all temperatures. More significant was the magnitude of the difference in the time required to attain a 5 log reductions in *L. monocytogenes* on the surface of bologna due to small difference in thickness. A difference of only 16 mm increased the time from 1.5 min to 9.5 min and 0.72 min to 4.12 min at 70 and 80°C, respectively.

Effect of surface application of nisin and/or lysozyme (5000 AU nisin/ml, 80 AU lysozyme/ml, and 5000 AU nisin + 80 AU lysozyme/ml) in combination with in-package pasteurization (60, 62.5 and 65°C) of RTE low fat turkey bologna on the inactivation of

L. monocytogenes was the second objective investigated. Nisin-lysozyme combination and nisin treatments were effective in reducing the time required for a targeted reduction in *L. monocytogenes* population at 62.5 and 65°C, but not at 60°C.

Finally, the ability of in-package pasteurization at 65°C for 32 s combined with pre-surface application of nisin and/or lysozyme (antimicrobial treatments were of the same concentration mentioned above) to reduce *L. monocytogenes* populations, and to prevent the subsequent recovery and growth during refrigerated storage for 12 weeks on the surface of low fat turkey bologna was determined. Nisin and nisin-lysozyme treatments were effective in reducing the growth of *L. monocytogenes* to below detectable levels by 2-3 weeks of storage.

DEDICATION

Dedicated to my parents and brothers who never demanded anything in return and to my wife Preetha who made this a reality.

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CHAPTER 1
INTRODUCTION

Microbiological safety of food is an important concern to the public as well as to the food industry. Foodborne illness resulting from consumption of food contaminated with pathogens is a major public health problem in the United States and in other nations of the world. Microbial contamination of foods has a serious impact on the food industry. Industry is often affected through severe economic losses resulting from food product recalls and also from plant closings and cleaning up operations associated with recalls.

In order to avoid the burden associated with food pathogens, food industry is constantly seeking new preventive measures to reduce contamination and novel preservation methods to eliminate these organisms from food products.

Listeria monocytogenes is a food pathogen which is able to withstand various environmental and processing stresses. The organism often inhabits the processing environment and thereby contaminates products after thermal processing. As a result, ready-to-eat foods (RTE) that are commonly consumed without further cooking are of particular concern. RTE meat products including poultry products have been associated with many listeriosis outbreaks. Majority of the food products involved in United States Department of Agriculture (USDA) recalls associated with *Listeria* spp. contamination were RTE meat and poultry products. In response, the USDA has implemented a "zero tolerance" for *L. monocytogenes* on RTE meat products.

For cooked meat products, contamination with *L. monocytogenes* occurs at the outer surfaces of the product during processing steps such as cutting or slicing before

packaging. *L. monocytogenes* can grow and multiply at refrigeration temperatures. Therefore, the growth of the organism in vacuum-packaged, cooked meat products can occur during storage and distribution. Additional processing treatments are needed before and after packaging to reduce the population and control the growth of the organism in RTE meat products during storage. The USDA has implemented many regulations and issued guidelines for the industry to prevent contamination and to eliminate *L. monocytogenes* from RTE meat and poultry products. Recently USDA has issued 3 alternatives for the industry which include use of a post-lethality treatment, antimicrobial agents, and sanitation measures to control *L. monocytogenes* in RTE products. Application of combined preservation methods, known as hurdle technology, is an efficient approach to improve the microbiological safety of food. Combining different preservative factors often will have a synergistic effect on restricting microbial growth.

Thermal processing is one of the most efficient methods for killing or reducing microbial populations in foods and in-package pasteurization is an effective method to reduce post-process contamination in RTE meat products. For developing an effective in-package pasteurization process for a specific meat product, the rate of inactivation of the target organism at specific temperatures needs to be studied. Meat product and package characteristics may influence the efficiency of in-package pasteurization in attaining targeted microbial lethality and therefore should be given special attention while developing an in- package pasteurization method for RTE meat products.

Another important factor to consider is the duration of in-package pasteurization. Since RTE meat products undergo an initial thermal treatment during cooking, the second heat treatment (in-package pasteurization) should be of minimum duration to conserve

the organoleptic qualities of the product. One practical approach to achieve this objective is to combine in-package pasteurization with other preservation methods in order to reduce the heat resistance of the organism and thereby reduce the magnitude of the heat treatment required.

Surface application of various antimicrobial agents alone as well as in combination is gaining attention as an effective method to reduce *L. monocytogenes* contamination. Nisin is a natural antimicrobial compound, which exerts a rapid bactericidal effect against gram-positive bacteria including *L. monocytogenes*. It is an FDA approved antimicrobial agent for foods. Nisin has been shown to be synergistic with some other antimicrobial agents and heat. Lysozyme is a natural enzyme used as a food preservative. Recent studies have shown that combining nisin and lysozyme has a synergistic effect on the resulting bactericidal activity of each component when used alone.

Based on the various aspects discussed above, the main objective of this study was to evaluate surface application of nisin and/or lysozyme in combination with in-package pasteurization of RTE low fat turkey bologna to eliminate *L. monocytogenes*, particularly to enhance the inactivation of the organism and to prevent its growth during storage. The effect of product thickness and composition on surface heating rate during in-package pasteurization was also investigated.

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CHAPTER 2
LITERATURE REVIEW

MICROBIOLOGICAL SAFETY OF FOOD

Foodborne pathogens

Foodborne illness caused through contamination of food products by pathogenic microorganisms is a major public health problem in the US and around the world (White et al., 2002). Epidemiology of microbial food-borne diseases has changed in the last decade due to high susceptibility of human population to diseases, changing life styles, and the emergence of newly recognized human pathogens (Altekruse and Swerdlow, 1996; Berkelman, 1994). Even in industrialized countries, one out of every three people has a food-borne microbial illness event every year (WHO, 2002). A broad spectrum of microbial pathogens can contaminate food and cause illness after they or their toxins are consumed. These foodborne pathogens include a variety of enteric bacteria, aerobes and anaerobes, viral pathogens, parasites, marine dinoflagellates, biotoxin producing bacteria in fish and shellfish, and prions (Tauxe, 2002). Some of the important foodborne bacteria are *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*, *Clostridium botulinum*, *Clostridium perfringens*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Vibrio cholera*, and *Yersinia enterocolitica.* Important foodborne parasites include *Toxoplasma gondii*, *Trichinella*, *Taenia saginata, Taenia solium*, *Cryptosporidium parvum*, and *Cyclospora cayetanensis.* Important viruses causing foodborne diseases are Hepatitis A, Norwalk-like viruses, Astrovirus, and Rotavirus (Jay et al., 2005; Ray, 2004).

Every year, in the United States, foodborne infections cause millions of illnesses and thousands of deaths with most of the infections going undiagnosed and unreported (Tauxe, 1997). Annual costs of foodborne illness in the United States have been estimated between $10 and $83 billion (FDA-CFSAN, 2003). Foodborne illness poses a significant economic burden, damages consumer confidence and impacts international trading of food products (Glynn et al., 2006). Worldwide, the number of cases of gastroenteritis associated with food pathogens is estimated to be between 68 million and 275 million annually (Naravaneni and Jamil, 2005). Two major sources of foodborne bacteria in meat and meat products are from the animal which carries pathogenic bacteria and the processing environment which harbors them. Human beings acts as a source of pathogenic bacteria indirectly by cross contamination (Borch and Arinder, 2002).

A substantial proportion of foodborne illness is attributable to improper in-home food handling, preparation, and consumption practices by consumers (CAST, 1994; Redmond and Griffith, 2003). Improper practices include inadequate cooking, cooling and storage of foods, cross-contamination of raw and cooked foods, inadequate personal hygiene, and consumption of raw and undercooked foods (CAST, 1994; Doyle et al., 2000). Awareness of foodborne pathogens among consumers is a very important factor in reducing foodborne illness. A survey based study conducted by Lin et al. (2005) found that 94% of the US consumers were aware of *Salmonella* spp. and 90% of *E. coli* as a problem in food. But only 32% of consumers were aware of *Listeria* spp. and 7% were aware of *Campylobacter* spp.

Development of antibiotic-resistant foodborne pathogens is another microbiological food safety issue (Doyle and Erickson, 2006; White et al., 2002). Several antibiotic-

resistant pathogens that have been associated with animals used for food and are of public health concern include ciprofloxacin-resistant *Campylobacter* spp, cephalosporin-resistant *Salmonella* spp and *E. coli*, multi-drug resistant a *Salmonella* Typhimurium DT104 and multi-drug resistant *Salmonella* Newport. In foodborne illnesses where antibiotic therapy may be necessary, patient recovery could be compromised by antimicrobial pathogen resistance (Doyle and Erickson, 2006).

Preservation methods to enhance microbiological safety

There are a number of conventional and new preservation technologies which are effectively used to control the growth and multiplication of pathogenic organisms in food. Thermal pasteurization and sterilization are predominantly used in food industry for their efficacy and product safety (Lado and Yousef, 2002). Advances in technology have allowed the optimization of thermal processing for maximum efficacy against microorganisms and minimum deterioration of food quality (Lado and Yousef, 2002).

The most publicized new preservation methods include non-thermal processes like high pressure processing (HPP), pulsed electric fields (PEF), irradiation, new packaging systems such as modified atmosphere packaging (MAP) and active packaging, natural antimicrobial compounds and biopreservation (Devlieghere et al., 2004).

Another food preservation approach that is being extensively studied is the 'hurdle technology' concept (Leistner and Gorris, 1995; Leistner, 2000). Hurdle technology uses the deliberate combination of existing and new preservation techniques in order to establish a series of hurdles that microorganisms present in the food must overcome to survive. Sometimes, because of their synergistic effect, the individual hurdles may be set at lower intensities than would be required if only a single hurdle were used as a preservative method (Leistner and Gorris, 1995). The most commonly applied hurdles in

food preservation include temperature, pH, redox potential, preservatives and competitive microorganisms (Leistner, 2000).

Developments in food safety management systems

The food supply chain is rapidly growing in size and diversity because of changes in lifestyle, demographic compositions and food market globalization. In order to keep pace with these changes in the food supply chain, it has been necessary to adapt and improve the food safety management systems on a continuous basis (Gorris, 2005). Hazard Analysis Critical Control Points (HACCP) and pre-requisite systems like Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) have been used as excellent tools in food safety management (van Schothorst, 2004). These systems are very specific to the food production facility for which they have been developed (Gorris, 2005). Considering the complex nature of the food chain, specific concepts have been developed recently in food safety management (Gorris, 2005). The acceptable level of a microbiological hazard is currently not often expressed in terms of its frequency and/or concentration, but just as the level which is as low as reasonably achievable (ALARA) by the industry (van Schothorst, 2005; Walls and Buchanan, 2005). The latest developments in food safety control advocate a shift from ALARA food safety management to a more risk based and targeted approach (van Schothorst, 2005). The International Commission on Microbiological Specifications for Foods (ICMSF, 2002) has proposed the establishment of Food Safety Objectives (FSO) to give more clear and firm guidance to food industries on the level of hazard deemed tolerable in a product at consumption. The FSO is defined as the maximum frequency and/or concentration of a microbial hazard in a food considered tolerable for consumer protection at the time of consumption (ICMSF,

2002). A hypothetical example for a FSO is "*L. monocytogenes* in a ready-to-eat food product shall not exceed 3.5 $\log_{10}$ cfu/serving size of food when eaten" (Gorris, 2005). Setting the FSO at the time of consumption requires consideration of the likelihood and impact of contamination at all points further back in the food chain (Walls and Buchanan, 2005). In order to meet the FSO, food chains need to employ a set of target points like Performance Objectives (PO) and Performance Criterion (PC) earlier in the supply chain (van Schothorst, 2005; Walls and Buchanan, 2005). Performance Objective is defined as the maximum frequency and/or concentration of a hazard in a food at a specified point in the food chain that should not be exceeded in order to achieve an FSO. If a ready-to-eat food product supports the growth of *L. monocytogenes* during normal refrigerated storage, the PO at the point of manufacture will be more stringent than the FSO to account for the potential growth of the organism during distribution and home use (Walls and Buchanan, 2005). Performance Criterion is defined as the effect in frequency and/or concentration of a hazard in a food that must be achieved by one or more control measures to contribute to a PO or an FSO (CAC, 2004). PC is the outcome of a processing step or a combination of steps to reduce the levels of a microorganism. A specific reduction of *L. monocytogenes* that is set for a post-lethality treatment of RTE meat products is an example for a PC.

The current health status of a population is evaluated by conducting a Microbiological Risk Assessment (MRA) which is composed of a Risk Assessment, Risk Management, and Risk Communication (Reji et al., 2004). The outcome of a risk analysis can be guidelines for handling food products or microbiological criteria that helps to minimize the number and impact of foodborne outbreaks (Reji et al., 2004). Epidemiologic data of

human disease, especially those from outbreak investigations, guide immediate preventive measures by contributing to assessing risk, helping to prioritize food safety problems, providing dose-response information from outbreak investigations, and validating risk estimates (ICMSF, 2006).

LISTERIA MONOCYTOGENES

Characteristics of the organism

L. monocytogenes is a gram-positive, motile, facultative anaerobic bacterium that is found in a broad ecologic niche. The organism is psychrophilic and takes advantage against other Gram-positive and Gram-negative microorganism in cold environments, such as refrigerators (Schlech, 2006). The organism can exist in an intracellular state within monocytes and neutrophils, and its name is derived from the fact that large numbers of monocytes are often found in peripheral blood of monogastric animals infected by this organism (Gray and Killinger, 1966). *L. monocytogenes* is able to initiate growth in the temperature range of 0 to 45°C. The average generation times for *L. monocytogenes* strains were 43, 6.6, and 1.1 h at 4, 10, and 37°C, and respective lag times were 151, 48, and 7.3 h (Barbosa et al., 1994). Although *L. monocytogenes* grows best in the pH range of 6-8, the organism can initiate the growth in laboratory media at pH values as low as 4.4 (Lou and Yousef, 1999). The organism grows optimally at water activity (a_w) $\geq$ 0.97. For most strains the minimum a_w for growth is 0.93, but some strains may grow at a_w values as low as 0.90 (Lou and Yousef, 1999). *L. monocytogenes* is able to grow in the presence of 10 to 12% of sodium chloride. The bacterium survives for long periods in high salt concentrations and the survival is significantly increased by lowering the temperature (Swaminathan, 2001).The nutritional requirements of *Listeriae* are

typical of those for many other Gram-positive bacteria. They grow well in many common media such as brain heart infusion, trypticase soy, and tryptose broth (Jay et al., 2005).

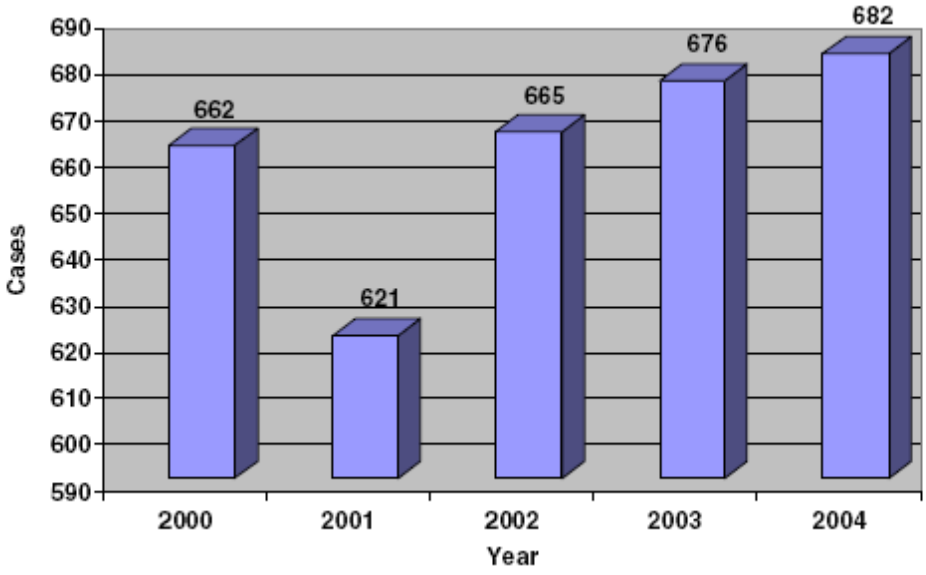
Public health significance

L. monocytogenes causes a foodborne disease, listeriosis which is recognized as an important worldwide public health problem. Incidence of listeriosis in developed countries ranges from 4 to 8 cases per 1,000,000 individuals (FAO/WHO, 2000). Due to its severe character, the hospitalization rate for listeriosis is 92%, while the case fatality rate is 20% (Mead et al., 1999). Almost all listeriosis cases (99%) have a foodborne source. Human listeriosis, although an uncommon disease, accounts for approximately 28% of the estimated annual disease deaths in the United States that are associated with a known pathogen (Mead et al**.,** 1999). The population groups most commonly affected by listeriosis are pregnant women, neonates, the elderly, and people with suppression of immune system, such as AIDS, cancer, or transplant patients (Gerba et al., 1996). Severe cases of listeriosis are often manifested as septicemia and / or meningoencephalitis. The mortality rate associated with listeriosis is on average 30% compared to a 0.38% and 0.1% for Salmonellosis and Campylobacteriosis, respectively, which makes listeriosis a serious public health problem (Wing and Gregory, 2002).

The first confirmed foodborne outbreak of listeriosis was associated with consumption of locally prepared coleslaw in 1981 in Nova Scotia, Canada. Thirty four pregnancy associated cases and seven cases in non-pregnant adults occurred during a 6 month period (Swaminathan, 2001). Pasteurized milk was identified as the most likely source of infection in another large outbreak of listeriosis in Boston, Massachusetts, in 1983 involving 42 immunosuppressed adults and 7 pregnant women (Fleming et al., 1985). In December 1998, an outbreak of invasive listeriosis was reported through

consumption of hot dogs and processed meats produced by a firm in which over 100 people became ill and 20 died (Wing and Gregory, 2002). A multistate outbreak of listeriosis occurred from May to November 2000 and was attributed to delicatessen meat (CDC, 2000). In 2002, a major outbreak of listeriosis in northeastern United States associated with consumption of sliceable turkey deli meat resulted in 46 cases including 7 deaths (CDC, 2002). Mead et al. (2006) described a nation wide outbreak of listeriosis involving residents of 24 US states. They used molecular subtyping to investigate the outbreak and suggested that *L. monocytogenes* strains vary widely in virulence and confirmed that large outbreaks can occur even when only low levels of contamination was detected in sample foods. Public health surveillance, outbreak investigations, and applied and basic research conducted during past two decades have helped characterize listeriosis, define the magnitude of its public health problem, determine its impact on the food industry, identify the risk factors associated with the disease, and develop appropriate control strategies (Swaminathan, 2001).

Fig. 2.1. Annual *Listeria* cases 2000 – 2004 (CDC, 2005).



Contamination of Ready-to-Eat (RTE) meat and poultry products

RTE meat and poultry products are products that are in a form that is edible without additional preparation but may receive additional preparation for palatability or aesthetic, gastronomic, or culinary purposes (FSIS, 2003a). RTE foods including red meats, poultry, seafood and vegetables have been documented as vehicles for several bacterial pathogens resulting in foodborne outbreaks (Gibbons et al., 2006). *L .monocytogenes* has been recovered from RTE meats worldwide due to its ability to survive and multiply in vacuum and gas-packaged products at refrigeration temperatures (Duffy et al., 1994). Post-processing contamination during steps such as slicing, peeling and packaging is the main route through which *Listeria* spp. contaminates RTE meat products (Wang and Muriana, 1994). The initial phase of contamination of these meat surfaces is presumably bacterial attachment followed by subsequent survival and growth (Dickson, 1991). Because of the ability of *L. monocytogenes* to multiply at refrigeration temperatures in some food products, even low-level contamination of a ready- to-eat product could result in substantial numbers of *L. monocytogenes* eventually being ingested by a susceptible individual (Seman et al., 2002). In 1989, the USDA implemented a zero-tolerance policy for *L. monocytogenes* in ready-to-eat meats (USDA, 1989). The immediate reason for implementation of this zero-tolerance policy was a clear link, established in 1989, between human listeriosis and a plant that produced turkey frankfurters (Wenger et al., 1990; CDC, 1989). As a result of this policy, current United States rules require that there be no detectable *L. monocytogenes* colonies within 25 g sample of RTE food product (Kathariou, 2002; Tompkin, 2002). However, complete elimination of *L. monocytogenes* from food products and prevention of post-processing contamination remains a challenge for food manufacturers.

The source and mechanism of contamination of RTE meat and poultry products by *L. monocytogenes* was investigated by several research workers. Cross-contamination between processing equipment and deli meats by *L. monocytogenes* was studied by Lin et al. (2006). The commercial slicer blade was inoculated with the organism and then meat samples were sliced and five consecutive meat slices were packed per package, vacuum sealed, stored at 4°C, and sampled at 1 and 30 days post-slicing. They found that the organism could be transferred from a contaminated slicer onto meats and can survive or grow on deli meats with preservatives. Higher *L. monocytogenes* cell numbers inoculated on the slicer blade resulted in more *L. monocytogenes* positive sliced meat samples.

Attachment of bacterial cells to surfaces can be affected by cell surface charge; hydrophobicity; hydrophilicity; steric hindrance and roughness (Cunliffe et al., 1999). The mechanism of attachment of five strains *L. monocytogenes* in a mixed cocktail to frankfurters, ham, and bologna was studied by Foong and Dickson (2004). They found that approximately 84 to 87% *L. monocytogenes* were found to strongly attach on to RTE meats within 5 min regardless of strain or meat type. No differences were observed in cell surface charge or cell surface hydrophobicity among strains.

Control strategies are needed at certain stages from pre-harvest to consumption (during processing, at retail and food service, and in the home) to minimize the likelihood that food will become contaminated with *L. monocytogenes* and to prevent the growth of the organism to high numbers (ILSI Research Foundation, 2005). Tompkin (2002) provided guidance to food processors in controlling *L. monocytogenes* in processing environments which included prevention of the establishment and growth of the organism in sites that can lead to contamination of RTE foods. Implementation of a sampling

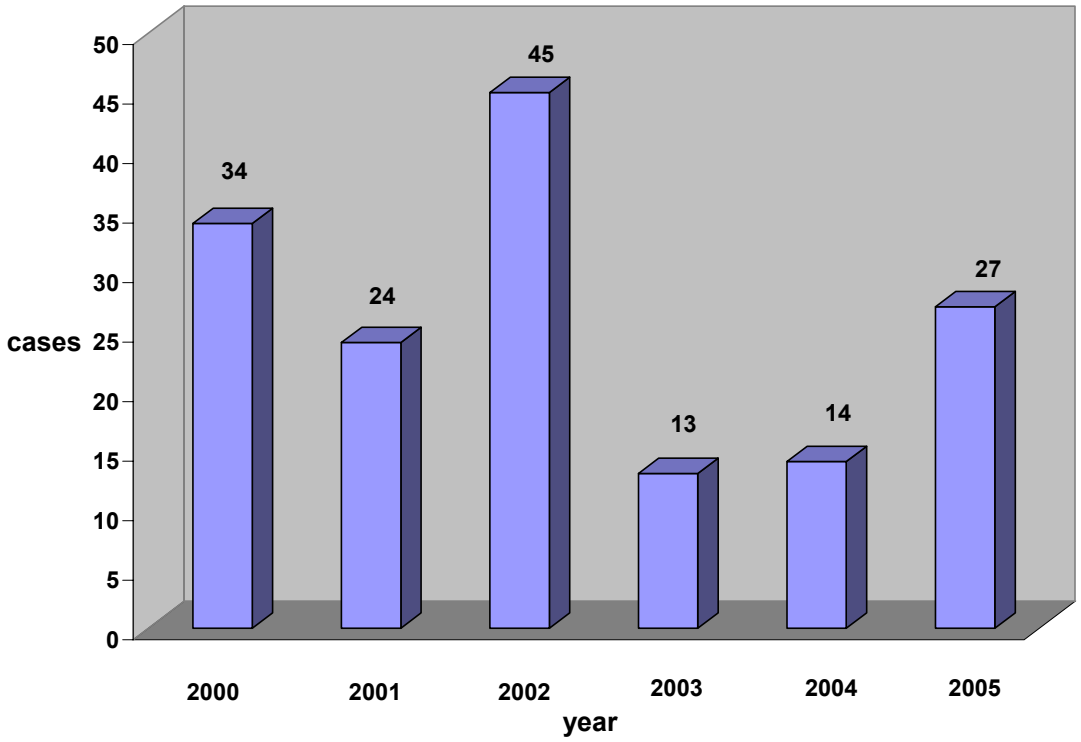
program that can assess in a timely manner whether the environment to which RTE foods are exposed is under control and can verify by follow-up sampling that a source of contamination has been detected and corrected. Porto et al. (2004) studied the effect of reheating on viability of a five strain mixture of *L. monocytogenes* in vacuum-sealed packages of frankfurters following refrigerated or frozen storage. These researchers found about a 5-log reduction was achieved by reheating to a surface temperature of 70°C for about 2 min or 80 or 90°C for about 0.6 min regardless of storage conditions or formulations. Proper storage and handling of refrigerated RTE meats can help reduce the risk of listeriosis. Consumer knowledge on storage, and handling practices regarding *Listeria* spp. in frankfurters and deli meats was evaluated by Cates et al. (2006) through a web-based survey. Despite limited awareness of *Listeria* spp. (44% compared to 94% for *Salmonella* spp. and *E. coli*) responses found many people were following recommended storage guidelines for frankfurters and deli meats. They also found that most individuals who were aware of *Listeria* spp. had limited knowledge about the pathogen and were unable to identify possible food vehicles. Thus, there is a need to educate consumers about the possible sources of *Listeria* spp. food products and proper handling and storage of RTE foods such as frankfurters and deli meats to help prevent listeriosis.

Impact on the food industry

As a result of the regulatory attention that RTE food products have received based on the "zero tolerance" policy for the presence of *L .monocytogenes*, there have been significant numbers of *Listeria*-related recalls (Marsden et al., 2001). The negative impact of a product recall may affect consumer demand for the product involved, leading to millions in lost sales, as well as loss of brand equity (Ivanek et al., 2004). Meat and poultry recalls have a direct economic and public perception impacts on the food industry

(Kramer et al., 2005). Research has shown that when meat recalls are announced there is a direct negative effect on demand for meat products with an associated move towards non-meat products (Marsh et al., 2004). A product recall is an action taken voluntarily by food manufacturers or distributors after they determine independently or are informed by a government agency of the possibility of negative health concerns for consumers from eating their products. Recalls are initiated by the manufacturer or distributor of the meat or poultry, sometimes at the request of FSIS. All recalls are voluntary. However, if a company refuses to recall its products, then FSIS has the legal authority to detain and seize those products in commerce (FSIS, 2002). The purpose of the recall is to effectively remove meat, poultry, or egg products which are believed to be adulterated or misbranded from commerce (Teratanavat and Hooker, 2004). Additional expenses incurred to the industry due to *L. monocytogenes* contamination include plant closings, clean up after recall, product liability costs, and insurance administration costs (Ivanek et al., 2004). Preventive control measures against *L. monocytogenes* contamination of RTE meat products require extra work, cleaning, disinfection, alteration of production procedures, and education, which all cost money. Some of these costs are reflected in changing stock market prices for the meat industry (Thomsen and McKenzie, 2001).

Fig. 2.2. Listeria related food product recalls – 2000-2005 (Source: FSIS recall center).



USDA –FSIS regulations

In the US, the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) is responsible for ensuring safe meat, poultry, and pasteurized egg products produced in Federally Inspected Plants (FIP) (USDA, 2000). The Food and Drug Administration (FDA) and FSIS have unrestricted enforcement authority to selectively sample and test for *L. monocytogenes* (Shank et al., 1996). FSIS designed the regulations to encourage establishments to employ more effective *Listeria* control measures. All establishments that produce RTE products that are exposed to the environment after lethal treatments will be required to develop written program, such as Hazard Analysis and Critical Control Points (HACCP) systems, Sanitation Standard Operating Procedures (Sanitation SOPs) or other programs to control *L. monocytogenes*

(FSIS, 2003b). In 2001, FSIS issued a proposed RTE rule (66 FR 12590) to require that all establishments producing RTE meat and poultry products conduct environmental testing of food contact surfaces for *Listeria* spp. after lethality treatment and before final product packaging (FSIS, 2001). Data gathered during an outbreak of *Listeria* related illness during the summer of 2002, combined with other food safety investigations and in-depth verification reviews, led FSIS to conclude that some establishments were not adequately addressing the potential for bacterial contamination in their HACCP plans, Sanitation SOPs or other control measures (FSIS, 2003b). In December 2002, FSIS implemented a directive outlining additional steps to be taken by USDA inspectors to ensure that establishments producing RTE meat and poultry products are preventing *L. monocytogenes* contamination. Under this directive, plants producing deli meats and hot dogs without validated *Listeria* programs to eliminate the organism on the product, on food contact surfaces, and in the environment were subject to an intensified FSIS testing program (FSIS, 2003b). In 2003, FSIS released compliance guidelines to control *L. monocytogenes* in post-lethality exposed RTE meat and poultry products and updated these guidelines in May 2006 (FSIS, 2006). The interim final rule (9CFR 430) includes three alternative approaches that establishments can take in the processing of RTE meat and poultry products during post-lethality exposure. Alternative 1 requires the use of a post-lethality treatment to reduce or eliminate *L. monocytogenes* and an antimicrobial agent or process to suppress or limit the growth of the pathogen. An establishment that identifies its products in Alternative 2 must apply either a post lethality treatment or an antimicrobial agent or process that controls the growth of *L. monocytogenes.* Under Alternative 3, the establishment does not apply a post-lethality treatment or an

antimicrobial agent or process to control the growth of *L. monocytogenes* in the post-lethality exposed product, instead must control the pathogen in its post-lethality processing environment through the use of sanitation control measures, which may be incorporated in the establishment's HACCP plan, Sanitation SOP or prerequisite program (FSIS, 2006). In the first two alternative approaches, the establishment must validate the use of post-lethality treatment or antimicrobial agent for its specific products. In case of using third alternative, since the establishment is not relying upon a post-lethality treatment or an antimicrobial agent or process to control *L. monocytogenes*, the product will be subjected to more frequent FSIS verification testing compared to the other alternatives.

Continued efforts from Government regulatory agencies, researchers, and manufacturers are needed to bring the overall level of food-borne listeriosis to the USA Healthy People 2010 goal of 0.25 cases per 100,000 individuals per year (DHHS, 2000).

Heat resistance

Thermal treatment destroys foodborne pathogens and is one of the primary techniques employed to ensure the safety of foods. Therefore, the survival and heat resistance of *L. monocytogenes* under a variety of conditions in many foods have been investigated. Heat resistance of *L. monocytognes* is influenced by many factors such as strain, previous growth conditions, exposure to heat shock, acidity, other stresses, and composition of the heating menstruum (Doyle et al., 2001). Results from numerous studies indicate that *L. monocytogenes* is more resistant to heat when tested in foods than when it is suspended in laboratory media (Boyle et al., 1990; Cassadei et al., 1998). High fat (30.5%) ground beef was more protective of *L. monocytogenes* at 57.2 and 62.8°C than low fat (2%) beef as indicated by higher D values of 5.8 vs. 2.6 and 1.2 vs. 0.6 min, respectively (Fain et al.,

1991). The effect of heat shock on thermal resistance of *L. monocytogenes* in meat was investigated by Farber and Brown (1990). A sausage mix inoculated with approximately 10^7 cfu of *L. monocytogenes* / g was initially subjected to a heat shock temperature of 48°C before being heated at a final test temperature of 62 or 64°C. Cells heat shocked for 120 min showed an average 2.4-fold increase in the $D_{64°C}$ value. Heat-shocked cells shifted to 4°C appeared to maintain their thermotolerance for at least 24 h after heat shock. Juneja et al. (1998) studied the effect of pH, acidulant, and growth temperature on the heat resistance of *L. monocytogenes* in brain heart infusion broth acidified to pH 5.4 or 7 with either lactic or acetic acid. The thermal resistance of cells cultured at a particular temperature was significantly lower when lactic acid was used to acidify the medium to pH 5.4. Regardless of acid identity, D values significantly decreased with increased growth temperature when the pH of the growth medium was 5.4, whereas D values significantly increased with increased temperature at pH 7. These results indicate that the heat resistance of *L. monocytogenes* depends upon its growth conditions. Slow heating (1.3°C/min) of inoculated ground pork samples allowed survival of more *L. monocytogenes* than rapid heating (8.0°C/min). More survivors were also detected in pork that was heated aerobically rather than anaerobically (Kim et al., 1994). Addition of curing salts to beef and pork enhances the thermotolerance of *L. monocytogenes* by two- to eightfold (Farber, 1989; Mackey et al., 1990). Further experiments determined that this protective effect was primarily due to NaCl and not due to the addition of fat often added to sausage or to sodium nitrite, sodium lactate, or sodium erythorbate (Mackey et al., 1990; Yen et al., 1991). The influence of a_w lowering ingredients such as salt and sugar on thermal resistance in yolk was investigated by Palumbo et al. (1995) using a five strain

mixture of *L. monocytogenes.* The D value for *L. monocytogenes* at 64.4°C increased from 0.44 min in plain yolk to 8.26 min after a 21.5 min lag in yolk with 10% salt and 5% sugar, and 27.3 min after a 10.5 min lag in yolk with 20% salt. Thus a A_w lowering solutes in liquid yolk increased the thermal resistance of *L. monocytogenes*.

NISIN

General Properties

Nisin is the most commonly used natural antimicrobial compound which was approved for use in food in 1969 and was awarded generally recognized as safe (GRAS) status in the United States in 1988 (FDA, 1988). Nisin is effective in a number of food systems, inhibiting the growth of a wide range of Gram-positive bacteria, including many important foodborne pathogens such as *L. monocytogenes* (Tagg et al., 1976). Nisin is a low- molecular- weight, heat stable and non-toxic polypeptide produced by the bacterial starter culture *Lactococcus lactis* subspecies *lactis*. The polypeptide can be prepared from culture fluids or the cells of the producer organism. Nisin is a 34-amino acid polypeptide with a molecular mass of 3510 Daltons. Nisin contains the thioether amino acids lanthionine and β-methyllanthionine, as well as amino butyric acid, dehydroalanine, and dehydrobutyrine (Thomas and Delves-Broughton, 2005). Nisin is a cationic molecule due to the combination of three lysine residues and one or more histidine residues together with lack of glutamate and aspartate. Nisin is most soluble at acidic pH, and becomes less soluble with increasing neutrality (Thomas et al., 2000).

Mode of action

Nisin is predominantly active against Gram-positive bacteria. By providing an induced damage of the outer membrane by some chelating agents, nisin can also made to act against Gram-negative bacteria (Ray, 1993). In a vegetative bacterial cell, the primary

site of action for nisin is the cytoplasmic membrane. Nisin produces pores on the membrane, which results in the rapid efflux of small cytoplasmic compounds like amino acids, potassium, inorganic phosphate, glutamate and ATP (Abee et al., 1994). The increase in membrane permeability results in the collapse of vital ion gradients and in complete dissipation of components of the proton motive force (PMF), transmembrane potential and pH gradient, leading to a rapid cessation of all biosynthetic processes (Bruno and Montville, 1993; Ruhr and Sahl, 1985). Collapse of the PMF leads to cell death through cessation of energy requiring reactions (Bauer and Dicks, 2005). Nisin is found to inhibit bacterial cell wall biosynthesis in vitro through a slow process (Linnet and Strominger, 1973; Reisinger et al., 1980). Nisin may also induce autolysis of susceptible staphylococcal cells (Bierbaum and Sahl, 1985). The peptides replace lytic enzymes from their cell wall intrinsic inhibitors. This apparent non-specific activation of cell wall hydrolysis by nisin results in extensive cell wall degradation, particularly in the septum area between dividing daughter cells. Cell lysis is encouraged by a combination of increased osmotic pressure and a weakened cell wall which results in pore formation (Bauer and Dicks, 2005). Spore-forming bacilli and clostridia and their spores are sensitive to nisin. Nisin inhibits the post-generation swelling and subsequent stages of spore development (Gould, 1964; Hitchins et al., 1963). This action may be due to the modification of sulfhydryl groups in the envelopes of germinated spores by nisin's dehydro residues (Morris et al., 1984; Montville et al., 1995).

Synergistic antibacterial activity with other compounds and methods.

Nisin is shown to have synergistic antibacterial activity with several preservative compounds and processes. This property makes nisin an ideal compound for use as a part of a multiple hurdle preservation program. One advantage of using nisin in combination

with other microbial hurdles is that nisin concentration required to have a targeted reduction can be lowered and thereby processor may be able to reduce the costs involved in using high concentrations of nisin. Another advantage of using nisin in combination with other methods or compounds is that it can be a way to overcome the problems of survival and growth of nisin resistant cells (Crandall and Montville, 1998).

Physical methods:

Heat:

Nisin is a heat stable bacteriocin which is reported as heat-stable at 100°C for 100 min (Mahadeo, 1995). This property is very useful in using nisin along with thermal preservation methods without inactivation of the compound. Various studies have shown the synergistic effect of nisin and heat in inactivating spoilage as well as pathogenic bacteria. The synergy is explained by heat induced changes in membrane permeability acting in concert with nisin-induced changes in membrane composition to facilitate poration that leads to rapid cell death (Ueckert et al., 1998; Mazzotta and Montville, 1997; Winkowski et al., 1994). Mahadeo and Tatini (1994) studied the effect of a combination of nisin (100 IU/ml) and heat (52°C for 3min) on scald water samples and found that it had lower microbial counts when compared with those with either nisin or heating alone. They also reported the same trend in *L. monocytogenes* populations inoculated into scald water samples. Budu-Amako et al. (1999) investigated the synergistic effect of nisin and moderate heat on the reduction of *L. monocytogenes* populations in cans of cold-packed lobster. Addition of nisin at a level of 25mg/kg of can contents to the brine surrounding the lobster, in combination with a heat process giving internal can temperatures of 60°C for 5 min and 65°C for 2min, resulted in 3-5 log

reductions whereas heat or nisin alone resulted in decimal reductions of 1 to 3 logs. D values were determined for *Bacillus cereus* T spores and *B. stearothermophilus* spores in skim milk supplemented with various concentrations (0, 2000, 4000 IU/ml) of nisin using an immersed, sealed capillary tube procedure (Wandling et al., 1999). These researchers found that for both organisms, the addition of nisin lowered the apparent D values. They also suggested that spore control is likely due to enhanced sensitivity of spores to heat and the presence of residual nisin in the recovery medium that could prevent outgrowth of survivors. Knight et al. (1999) determined the D- values and z-values for *L. monocytogenes* in liquid whole egg with nisin and NaCl by a submerged glass ampoule procedure. Nisin significantly decreased D-values at lower (< 58º C) temperatures in both unsalted and salted liquid whole egg but had little effect on minimum US pasteurization temperatures of 60º C without NaCl and 63º C with NaCl. However, addition of nisin 2 h prior to heat treatment reduced the D values significantly at these temperatures. Ueckert et al. (1998) studied the synergistic antibacterial action of mild heat (48 and 56°C) in combination with low concentrations of nisin (4 IU and 20 IU/ml) on *Lactobacillus plantarum* and reported synergistic reduction in viability. The synergistic effect of heat treatment (55°C) and nisin (500 IU/ml) on nisin resistant and wild-type *L. monocytogenes* Scott A was studied by Modi et al. (2000). When nisin resistant cells were grown in presence of nisin, they were more sensitive to heat than wild-type cells with a D-value of 2.88 min and 3.72 min respectively. When nisin resistant cells were subjected to a combined treatment of heat and nisin, there was approximately a 4 log reduction during the first 7 min of treatment. Synergistic effect of nisin and heat treatment on the growth of *Escherichia coli* O157:H7 was determined in vitro by Lee et al. (2002). They found

that addition of 100 IU/ml nisin into the plates containing the organism which underwent a heat treatment of 50 and 52.5ºC for 15 min significantly inhibited *E .coli* growth but not with shorter heating times and lower temperatures.

High pressure processing:

High pressure processing (HPP) is an alternative nonthermal food preservation method to avoid post-processing contamination, especially for foods having thermosensitive nutritional, sensory, and functional properties (Aymerich et al., 2005). Nisin is found to have a good synergistic effect with HPP against various food pathogens. HPP kills or sub lethally injures cells by disruption of the cell wall and membrane, dissociation of protein and ribosomal subunit structures, and loss of activity of some enzymes (Hoover et al., 1989). This altered cell permeability will facilitate the enhanced activity of nisin. Both Gram-positive bacteria (*L. monocytogenes*) and Gram-negative bacteria (*E .coli* and *S*. Typhimurium) showed increased levels of inactivation when nisin was present during pressure treatment (Kalchayanand et al., 1994). Nisin in combination with high-pressure treatment showed strong synergistic effects against *L. plantarum* and *E. coli* at temperatures less than 15°C (ter Steeg et al., 1999). Elimination of both organisms was achieved at 10°C with synergistic combination of nisin (4 IU/ml for *L. plantarum* and 80 IU/ml for *E. coli*) at 200 MPa for 10 min. Addition of nisin (5mg/l) and HPP treatment (450 MPa) of liquid whole egg resulted in 5 log reductions of *E. coli* and 6 log reductions in *L .innocua* (Ponce et al., 1998). This treatment resulted in complete elimination of both organisms after 1 month of storage at 4°C. The combined effect of high pressure, nisin and acidification on the mesophilic and psychrotrophic bacteria of mechanically recovered poultry meat was evaluated by Yuste et al. (2002). Significantly

highest decrease (5.3 log cfu/g for mesophiles and above 7.5 log cfu/g for psychrotrophs) in populations occurred in samples with 200 ppm of nisin treated at 450 MPa pressure.

Kalchayanand et al. (2004) studied the viability loss of *L. monocytogenes, Salmonella* Typhimurium, and *E. coli* O157:H7 by hydrostatic pressure in the presence and absence of a combination of nisin and pediocin. There was a significant reduction in bacterial counts by using the combination of high pressure and bacteriocin mixture compared to the use of the methods individually.

Pulsed electric fields (PEF):

The use of shorter duration high voltage pulses for non-thermal inactivation of microorganisms in food is a new preservative method. The bactericidal effect of PEF treatment is believed to be based on membrane electrocompression as a result of induced transmembrane potential. When this transmembrane potential reaches about 1 V (natural potential of the cell membrane), the electrocompressive force exceeds the elasticity of the membrane resulting in pore formation (Sale and Hamilton, 1968; Zimmermann, 1986). The combination of PEF and nisin treatments could be a means of achieving the desired level of microbial inactivation since both acts on destabilizing the cell membrane (Dutreux et al., 2000). Pol et al. (2000) reported inactivation of vegetative cells of *B. cereus* by a combined low dose of nisin (0.06μg/ml or 2.4 IU/ml) and mild PEF (16.7Kv/cm, 50 pulses each of 2 μs) by an additional 1.8 log cfu /ml greater than the sum of reductions obtained with single treatment. The exposure of *L. innocua* to nisin after PEF in skim milk had an additive effect on the inactivation of microorganism compared to that exhibited by the PEF alone (Calderon-Miranda et al., 1999a). Reduction of the organism as a result of exposure to 10 IU nisin /ml after 32 pulsed electric fields was 2,

2.7, and 3.4 logs for an electric field intensity of 30, 40, and 50kV/cm, respectively. The same research group (Calderon-Miranda et al.,1999b) found that in liquid whole egg, *L. innocua* exposed to 10 IU nisin /ml after PEF exhibited a 4.1 log reduction for an electric filed intensity of 50kV/cm and 32 pulses. Death and injury of *Micrococcus leuteus* following exposure to nisin and PEF were investigated in phosphate buffer by Dutreux et al. (2000) and found that application of nisin clearly enhanced the lethal effect of PEF treatment. PEF treatment (50 pulses at 33 kV/cm) followed by nisin (100 IU/ml) caused of 5.2 log reduction in comparison with a 4.9 log reduction obtained with nisin followed by PEF.

Incorporation into packaging films:

The use of packaging films as antimicrobial delivery systems to reduce spoilage and pathogenic bacteria has been extensively studied. Various approaches have been proposed and demonstrated for the use of edible and polymer films to deliver bacteriocins, such as nisin, to a variety of food surfaces including muscle foods (Cutter et al., 2001). Padgett et al. (1998) tested packaging films with nisin or lysozyme incorporated into the film structures (produced by two methods- heat press and casting) separately for inhibition against *L. plantarum* and both compounds in combination with EDTA in films against *E. coli*. The minimum concentration of nisin (as Nisaplin™) that was effective in heat-press soy film was 0.1 mg/g of film, and as the concentration increased from 0.1 to 6.0 mg of nisin/g of film, the inhibition increased. Nisin concentration of 6 and 40 mg/g of film in heat-press corn zein films demonstrated inhibition with higher concentration showing more effect. Natarajan and Sheldon (2000) investigated the efficacy of polymer packaging films treated with purified nisin-

containing formulations to reduce *Salmonella* contamination of fresh broiler drumstick skin. Polyvinyl chloride, linear low density polyethylene, and nylon films were coated with 100μg/ml of nisin and varying concentrations of citric acid, EDTA, and Tween 80. Log reductions of 0.4 to 2.1 were obtained in *Salmonella* Typhimurium populations. Ko et al. (2001) studied physical and chemical properties of edible films containing nisin and their action against *L. monocytogenes*. As the nisin concentration increased (4.0-160 IU/ film disk), the amount of inhibition progressively increased in all tested films. In a series of five experiments, EDTA, lauric acid (LA), nisin, and the combinations of three antimicrobial agents were incorporated into a corn zein film and exposed to broth cultures of *L. monocytogenes* and *Salmonella* Enteritidis (Hoffman et al., 2001). There was a 4 log reduction in *L. monocytogenes* counts after 48 h exposure to films containing lauric acid and nisin alone. Of all film agent combination tested, none had greater than a 1 log reduction of *S.* Enteritidis when a10^8 cfu/ml initial inoculums were used. Effectiveness of packaging films coated with a methylcellulose/hydroxypropyl methylcellulose-based solution containing 10000, 7500, 2500, or 156.3 IU/ml nisin for controlling *L. monocytogenes* on the surfaces of vacuum-packaged hot dogs were investigated by Franklin et al. (2004). Packaging films coated with a cellulose-based solution containing 10, 000 and 7, 500 IU/ml nisin significantly decreased *L. monocytogenes* populations on the surface of hot dogs by greater than 2 log cfu/package throughout the 60-day study.

Diffusivity of nisin impregnated corn zein and wheat gluten films into water were affected by film type (wheat gluten or corn zein), environmental temperature and film forming methods (cast or heat-set) (Teerakarn et al., 2002). Cast wheat gluten film had

the greatest diffusivity while the cast corn zein film had the lowest and heat-pressed wheat gluten and corn zein films did not differ in diffusivity. Dawson et al. (2003) evaluated the effect of protein type (wheat or corn) and film forming method (casting or heat-pressing) on films for the retention of biologically active nisin and release of activity into water at four different temperatures (5, 25, 35, and 45°C). Cast corn zein and cast wheat gluten films retained 12.1% (8.1×10^4 IU/g film) and 15.8% (1.1×10^5 IU/g film) of the original activity after film formation, respectively. Heat-pressed corn zein and heat-pressed wheat gluten films retained 6.5% (4.3×10^4 IU/g film) and 7.4% (4.9×10^4IU/g film) of the original activity after film formation, respectively.

Nisin is sometimes adsorbed onto various surfaces and added to packaging films. Dawson et al. (2005) studied the antimicrobial activity of nisin-adsorbed silica and corn starch powders against *L. plantarum and L. monocytogenes* and found that nisin-adsorbed powders were highly efficient at both adsorption and release of antimicrobial activity.

Modified Atmosphere Packaging:

Modified Atmosphere Packaging (MAP) is one of the most investigated and effective food preservation methods. Carbon dioxide has a demonstrated antimicrobial activity among the gases used for MAP; however MAP alone may not be effective for use with cooked meat products to inhibit the growth of *L. monocytogenes* (Fang and Lin, 1994). The influence of carbon dioxide (CO_2) combined with various nisin concentrations on the growth of *L. monocytogenes* Scott A and *Pseudomonas fragi* on cooked tenderloin pork stored at 4 and 20°C was investigated by Fang and Lin (1994). Colony counts of *P. fragi* were appreciably reduced by MAP alone, but not for *L. monocytogenes*. Although *P. fragi* on cooked tenderloin was unaffected by 1×10^4 IU/ml of nisin, growth of *L.*

monocytogenes was prevented by the same concentration of nisin. But the MAP (100% CO_2, 80% CO_2 + 20% air) and nisin (103, 104 IU/ml) combination system decreased growth of both organisms, and this effect was more pronounced when samples were stored at 4 °C than at 20°C. The same authors (1994b) conducted a similar study to determine the effect of MAP (100% CO_2, 80% CO_2 + air) and nisin (0, 10^4, and 5×10^4 IU/ml) on the inactivation of *L. monocytogenes* on raw pork. In combination with nisin, 100% and 80% CO_2 were inhibitory to the growth of the organism. The combination treatment was also increasingly effective with increasing CO_2 and nisin concentrations.

Nilsson et al. (1997) investigated the inhibitory effect of nisin in combination with CO_2, NaCl and low temperature on the survival of *L. monocytogenes* in *in vitro* model studies, and in trials with cold-smoked salmon. The antilisterial effect of nisin was improved in the presence of 100% CO_2 and increasing NaCl concentrations (0.5 to 5.0% w/v). Addition of nisin (500 or 1000 IU/g) to cold-smoked salmon inoculated with *L. monocytogenes* and stored at 5°C, delayed, but did not prevent the growth of the organism in vacuum-packs. Addition of nisin to CO_2 packed cold-smoked salmon resulted in 1-2 log reduction of *L. monocytogenes* followed by a lag phase of 8 and 20 days with 500 and 100 IU nisin/g respectively. Mechanism of combined antilisterial effect of nisin and CO_2 was investigated by Nilsson et al. (2000). These researchers examined the synergistic action of CO_2 (100%) and nisin (2.5µg/ml) on *L. monocytogenes* Scott A wild type and nisin resistant cells grown in broth at 4°C. Nisin did not decrease the viability of nisin resistant cells however for wild-type cells an immediate 2-log reduction of viability was observed when they were grown in air and a 4-log reduction when they were grown in 100% CO_2. They attributed the enhanced lethal

action of nisin on cells grown in a CO_2 atmosphere to a change in cell membrane permeability by expanding the hydrophobic regions of the membrane and modification of their membrane fatty acid composition by increasing the short chain fatty acids at the expense of long chain fatty acids. A study on the combined effect of nisin, headspace CO_2 levels, and EDTA on the survival of *Pseudomonas aeruginosa* and *Enterococcus faecium* was carried out in a water-soluble fish muscle extract at 3°C (Cabo et al., 2001a). *E. faecium* was completely deactivated by all processing after 2 days of storage but *P. aeruginosa* was less susceptible to treatments. Cabo et al. (2001b) studied the effectiveness of CO_2 (30-90%) and Nisaplin (500-1500 mg/kg) on increasing shelf-life of fresh pizza. The combined use of Nisaplin and MAP lead to significant increases in shelf-life of commercially stored samples and this was ascribed to complementary effects of nisin and CO_2 against lactic acid bacteria and yeast.

Chemical preservative compounds:

Nisin is shown to have synergistic antimicrobial action with many chemical preservative agents including organic acids and their salts, chelating agents, and other chemical compounds. Nykanen et al. (2000) studied the inhibition of *L. monocytogenes* and mesophilic aerobic bacteria in cold-smoked rainbow trout by nisin (4000-6000 IU/ml), sodium lactate (60%) or their combination (1:1). 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 nisin and sodium lactate injected into smoked fish decreased the count of *L. monocytogenes* from 3.26 to 1.8 log cfu/g over 16 days of storage at 8 ºC. Effect of nisin or nisin combined with EDTA on the survival of *L. monocyogenes* and *E .coli* O157:H7 on vacuum packaged fresh beef was

evaluated by Zhang and Mustapha (1999). Treatment with nisin or with nisin combined with EDTA reduced the population of *L. monocyogenes* by 2.01 and 0.99 log cfu/cm^2 respectively as compared to control. The high pH of nisin-EDTA solutions must have contributed to the decreased activity of nisin and thereby lower reduction in microbial count. However, the effect of nisin and nisin combined with EDTA against *E .coli* O157:H7 was marginal at 1.02 log cfu/ cm^2. Hydrogen peroxide (2.5%) alone or hydrogen peroxide (1%) in combination with nisin (25µg/ml), sodium lactate (1%), and citric acid (0.5%) were investigated as potential sanitizers for reducing *E. coli* O157:H7 or *L. monocytogenes* populations on whole cantaloupe and honeydew melons by Ukuku et al. (2005). At days 0 and 7 melons treated with the combination were significantly lower in population for both pathogens, by 3 to 4 log cfu/cm^2 and the combination was more effective than with 2.5% hydrogen peroxide. A study by Long and Phillips (2003) investigated the effectiveness of sodium lactate (2% w/w), sodium citrate (1.5% w/w), and nisin (500 IU/g) singly as well as in combination on the survival of *Arcobacter butzleri* NCTC 12481 on chicken stored at 5 and 30°C. *A. butzleri* was insensitive to 500 IU/g of nisin at both temperatures while at 5°C, 2% sodium lactate alone, 2% sodium lactate + 500 IU/g nisin resulted in a statistically significant log reductions compared with a control (no antimicrobial treatment). The inhibitory effects of nisin (100 IU/ml) and monolaurin (250 µg/ml), used alone or in combination, were investigated against four *Bacillus* species as vegetative cells in milk at 37°C for 5 days by Mansour and Milliere (2001). Even though nisin induced an immediate reduction in population level, cell concentrations reached the control culture level because of recovery and regrowth. On the other hand, monolaurin had a sustained bacteriostatic effect followed by regrowth

to a level constantly lower than that of control culture. The combination of the two agents induced a synergistic bactericidal effect leading to total inhibition throughout 5 days except in case of *Bacillus cereus*. Samelis et al. (2005) evaluated dipping solutions of nisin (5000 IU/ml), with or without lactic or acetic acid (1, 3, 5g/100ml), sodium acetate or diacetate (3, 5g/100ml), and potassium benzoate or sorbate (3g/ml) as inhibitors of *L. monocytogenes* introduced on sliced cooked pork bologna before vacuum packaging and storage at 4°C for 120 days. Nisin alone reduced the organism by 1.0-1.5 log cfu/cm^2 at day-0 followed by a listeriostatic effect for 10 days. Nisin in combination with 3 or 5g/100 ml acetic acid or sodium diacetate or 3g/100 ml potassium benzoate did not permit growth before day-90.

Combined antimicrobial effect of nisin and other natural antimicrobial compounds like bacteriocins, plant extracts, animal derived compounds, fatty acids and their derivatives has been extensively studied. Inoculation studies on tofu prepared with nisin and protective cultures showed that lower amounts of nisin were required for an effective inhibition of *L. monocytogenes* when protective bacterial cultures of either *Enterococcus faecium* BFE 900-6a or *Lactococcus lactis* BFE 902 were used (Schillinger et al., 2001). The combination of nisin with these bacteriocinogenic lactic acid bacteria resulted in a complete suppression of listerial growth in homemade tofu stored at 10°C for 1 wk. Plant extracts intended for use as antioxidants in foods may also have bactericidal effects on bacteria. A bearberry (*Arctostaphylos uva-ursi*) leaf extract alone displayed no antimicrobial activity of its own but enhanced the activity of nisin against *Brochothrix thermosphacta* (Dykes et al., 2003). Thymol, a major essential oil component of thyme, has been tested for antibacterial effects against a wide range of organisms including *L.*

monocytogenes, *Staphylococcus aureus* and oral bacteria (Juven et al., 1994). Synergistic antimicrobial effects of nisin and thymol on the survival of *L. monocytogenes* and *Bacillus cereus* in broth was investigated by Ettayebi et al. (2000) who found that nisin activity was greatly enhanced by sub-inhibitory concentrations thymol. Paula and Moezelaar (2001) studied the combined effect of nisin and carvacrol, which is a phenolic compound present in the essential oil fraction of oreganum and thyme, at different pH and temperature levels on the viability of different strains of *B. cereus*. Carvacrol enhanced the inhibitory effect of nisin at lower pH values. Combination of nisin and lactoperoxidase system (LPS) showed a synergistic antimicrobial effect on *L. monocytogenes* in skim milk. The synergy was enhanced when nisin and LPS were added after 3 and 5 h of growth (Zapico et al., 1998). Bacteriophages form part of the microbiological flora of many foods and represent a potential natural mechanism to control bacteria that act as their host (Greer, 1986). The effect of nisin and listeriophage LH7, alone and in combination, on the growth and survival of two strains of *L. monocytognes* in broth and two model food systems was determined by Dykes and Moorehead (2002). The combination of the two compounds displayed an enhanced antibacterial effect in broth, but in model food systems, nisin alone had an effect and no combined action was found.

Synergy with lysozyme:

The antibacterial properties of lysozyme and nisin have been proven against Gram-positive bacteria. Lysozyme presently has a small number of applications in the food industry with the major usage involving the prevention of *Clostridium tyrobutyricum* spore outgrowth in hard cheeses (Wasserfall and Teuber, 1979). Combined effect of nisin

and lysozyme on different microorganisms has been reported by various researchers. Minimal inhibitory concentrations (MIC) of nisin and lysozyme mixtures (1:1 and 1:3) against lactic acid bacteria were found to be significantly greater than the parent molecules (Chung and Hancock, 2000). The authors pointed out the benefits of using the mixture against food spoilage bacteria, over the use of individual agents as 1) reducing the amount of expensive nisin by adding lysozyme which is 3-fold cheaper to the required quantity and 2) the ability of the combination to sustain activity even at high salt concentrations compared to a reduced activity of lysozyme. These researchers attributed the increased efficacy of the mixture to increased membrane damage, cell lysis, or the inhibition of energy dependent processes that repair nisin/lysozyme damage to the cell. The antimicrobials lysozyme, nisin, and the mixtures of the two were studied to ascertain their abilities to control the growth of the meat-borne spoilage bacteria, *Brochothrix thermosphacta* B2 and *Carnobacterium* sp.845 in APT broth, in a meat juice extract, and on cores of lean and fat tissue (Natress et al., 2001). These researchers hypothesized that lysozyme, in combination with nisin, at a higher concentration than 65μg/cm^2 might improve the antimicrobial activity of the mixture, as well as extend the time during which it would be effective. A mixture of nisin and lysozyme at a ratio of 1:3 (w/w) and at a surface concentration of 260 μg/cm^2 was effective in controlling the growth of lactic acid bacteria on naturally contaminated pork loins that were stored in vacuum packages at 2°C for up to 6 weeks (Nattress and Baker, 2003). But, interestingly the numbers of *Enterobacteriaceae* were higher in treated samples than untreated samples possibly due to the inhibition of lactic acid bacteria. A mixture of nisin and lysozyme (1:3) and EDTA were evaluated for the antibacterial effect on bologna and ham by incorporating the

agents as ingredients before cooking (500 mg/kg of nisin-lysozyme and 500 mg/kg of EDTA) as well as through surface application on the final product (25.5 g/liter of nisin-lysozyme plus 25.5 g/liter of EDTA) by Gill and Holley (2000 a, b) in two sets of experiments. In the first experiment where agents were used as ingredients, treatment reduced initial populations at different times of 4 weeks storage at 8°C. Treatment prevented the growth of *Brochothrix thermosphacta*, to week 4, *Lactobacillus curvatus* to week 3, *Leuconostoc mesenteroids* and *L. monocytogenes*, to week 2. In the second experiment, where antimicrobial treatments were applied to the surface in a 0.2 g of 7% gelatin, the treatment had an immediate bactericidal effect up to 4 log cfu/cm^2 on the four Gram-positive organisms tested (*B. thermosphacta, L. sakei, Lc. mesenteroids, and L. monocytogenes*) and inhibited the growth of these organism during 4 weeks of storage. The antimicrobial treatment also had a bactericidal effect on the growth of *Salmonella* Typhimurium during storage. In a study to investigate inactivation of high pressure resistant *E. coli* by lysozyme and nisin under pressure, Massachalk et al. (2000) found that a combination of nisin and lysozyme was better in reducing the tailing of high pressure survivor curves due to its ability to reduce the fraction of cells that survived the treatment compared to the use of nisin or lysozyme separately.

LYSOZYME

Antimicrobial enzymes play a significant role in the defense mechanisms of living organisms against infection by bacteria and fungi (Fuglsang et al., 1995). Lysozyme is an enzyme that belongs to a class of enzymes that lyse the cell walls of certain Gram-positive bacteria, as they split the bond between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in the bacterial cell wall. Lysozyme is widely

distributed in various biological fluids and tissues, including avian egg, plant and animal secretions, tears, saliva, and respiratory and cervical secretions, and is secreted by polymorphonuclear leukocytes (Jolles and Jolles, 1984). Lysozyme functions as a food preservative by destroying certain bacteria. Hen egg white lysozyme (HEWL) is one of the few natural antimicrobials derived from an animal source that are applied as a food preservative. It occurs naturally in high concentrations in eggs (up to 0.3 to 0.4 g per egg or 3% of the egg white protein) making it a low cost product (Masschalck and Michiels, 2003). Lysozyme has a long history of safe use as a natural food component, and the low, additional intake as a preservative was not considered as a hazard to consumer health. Therefore, it received the *generally recognized as safe* (GRAS) status by WHO/FDA (Masschalck and Michiels, 2003).

Physical and chemical characteristics

The molecular weight of chicken lysozyme is 14,307 and the isoelectric point is 10.7 (Losso et al., 2000). Lysozyme has four disulfide bonds, making the molecule unusually compact with high heat stablity. In dried powder forms, lysozyme can be stored for a long time (> 6months) at temperatures up to 30°C without losing lytic activity. Chicken lysozyme is stable at 100°C for 2 min at pH 4.5 and at 100°C for 30 min at pH 5.29 (Losso et al., 2000). Proteolytic enzymes (except for pepsin) such as trypsin, chymotrypsin, and papain do not hydrolyze native lysozyme; but these enzymes do hydrolyze denatured lysozyme. Lysoyme is inactivated by components of egg yolk such as lipovitellin (Proctor and Cunningham, 1988).

Mechanism of antimicrobial activity

Lysozyme is a peptidoglycan N-acetylmuramoylhydrolase and its natural substrate is peptidoglycan, also called murein, the major component of the bacterial cell wall

(Masschalck and Michiels, 2003). Gram-positive bacteria have a thick cell wall composed of up to 40 layers of peptidoglycan whereas Gram-negative bacteria typically have only a single layer of peptidoglycan that is, however, surrounded by an asymmetric bilayer membrane, called the outer membrane (Masschalck and Michiels, 2003). Therefore in Gram-negative bacteria, because the cell wall is protected by the outer membrane, it prevents lysozyme access to the cell wall. But Gram-negative bacteria were susceptible to lysozyme after the outer membrane of the bacteria had been disrupted by compounds such as EDTA, aprotinin, organic acids or when lysozyme was conjugated to carbohydrates (Johnson, 1994; Pellegrini et al., 1992). Lysozyme has been found to possess the ability to inactivate certain viruses, regardless of its enzymatic activity, by forming an insoluble complex (Hasselberger, 1978). Lysozyme's antibacterial property is mainly attributed to its catalytic (enzymatic) function on bacterial cell wall. But recently some researchers have found evidence that the antibacterial activity of lysozyme is independent of its enzymatic action (During et al., 1999; Ibrahim et al., 1996; Ibrahim et al., 2001). The evidence comes from the finding that the reduction or elimination of the enzymic activity of lysozyme by heat- or dithiothreitol denaturation or site directed mutagenesis did not necessarily reduce its bactericidal activity (Masschalck and Michiels, 2003).

Animicrobial activity against food spoilage and pathogenic bacteria

The most important commercial application of hen egg white lysozyme in the food industry is the prevention of late blowing in cheese production. Lysozyme prevents the growth of *Cl. tyrobutyricum* which will ferment lactate produced from lactose in the primary fermentation by the lactic acid starter bacteria instead, producing butyric acid, acetic acid, carbon dioxide and hydrogen causing the cheese to blow (Masschalck and

Michiels, 2003). A study by Wasserfall and Teuber (1979) found that a 500 AU/ml of egg white lysozyme was able to kill 99% of 5×10^5 resting vegetative cells of *Cl. tyrobutyricum* within 24 h of incubation at 25°C. But spores were completely resistant to lysozyme. Lysozyme was demonstrated to have antibacterial activity against organisms of concern in food safety, including *L. monocytogenes* and certain strains of *Cl. botulinum* (Hughey and Johnson, 1987). Antibacterial activity of HEWL against *L. monocytogenes* Scott A in various foods was evaluated by Hughey et al. (1987). They found that lysozyme was more active in vegetables than in animal-derived foods and maximum activity in some foods was obtained when they used lysozyme along with EDTA. Lysozyme, at 20 to 200mg/L delayed the growth of all four strains of *L. monocytogenes* isolated during a food poisoning outbreak (Johnson, 1994). The sensitivity of the pathogen to lysozyme depended mostly on the physiological state of the microbe and on the growth medium. Antimicrobial peptides released by enzymatic hydrolysis of HEWL showed bacteriostatic activity against Gram-positive bacteria (*Staphylococcus aureus* 23-394) and Gram-negative bacteria (*E. coli* K-12) (Mine et al., 2004). These peptides broadened the antimicrobial activity of lysozyme to include Gram-negative bacteria. Abdou et al. (2005) used commercially available lysozyme peptide preparations (100µg/ml) against *Bacillus* species and observed that the compound completely inhibited most of the organisms in the *Bacillus* species except *B. cereus* and *B. stearothermophilus* which showed slightly higher resistance.

Synergy with other antimicrobials

Lysozyme is not totally effective against all Gram-positive bacteria and is ineffective against most Gram-negative bacteria. Hurdle technology using lysozyme has shown significant improvement in lysozyme activity against wide range of bacteria (Losso et al.,

2000). Chelators are compounds that are added to sequester cations which destabilize the lipopolysaccharides of the outer membrane and allow the antimicrobials like lysozyme and nisin to penetrate the lipopolysaccharide layer, resulting in lysis of the cell (Boland et al., 2003). Razavi-Rohani and Griffiths (1996) studied the effect of lysozyme combined with ethylene diamine tetra acetic acid (EDTA) against 7 Gram-positive and 8 Gram-negative organisms. Lysozyme in combination with EDTA was found to be more effective than either agent alone against the majority of the organisms tested. The effectiveness of polyphosphates or lipases to increase the lytic activity of lysozyme was evaluated both on *L. monocytogenes* suspended in buffer and on growing cultures incubated at different temperatures (Liberti et al., 1996). At 5 and 37°C, polyphosphates combined with lysozyme did not result in a decrease in the number of non-growing *L. monocytogenes* cells. Under the same incubation conditions, the addition of lipase to lysozyme significantly enhanced the bactericidal activity of lysozyme to an extent determined by pH, NaCl concentration, and temperature. Carneiro del Melo et al. (1998) showed that use of low concentrations of (5mM) of trisodium phosphate sensitized *C. jejuni*, *E. coli*, *Psuedomonas fluorescens*, and *S.* Enteritidis for lysozyme. These researchers hypothesized that sublethal levels of trisodium phosphate may cause disruption of the outer membrane of Gram-negative bacteria. Ellison and Giehl (1991) observed that the combination of lysozyme and lactoferrin was synergistic and bactericidal for *Vibrio cholerae*, *S.* Typhimurium, and *E. coli.* Lactoferrin, a milk protein, damaged the outer membrane of Gram negative bacteria and thereby increased penetration of lysozyme through the outer membrane (Ellison and Giehl, 1991; Yamauchi et al., 1993). Helander et al. (1998) found that carvacrol, thymol, and trans-

cinnamaldehyde sensitized *E. coli* and *S*. Typhimurium to lysozyme. Malicki et al. (2004) evaluated the effect of 2% sodium lactate alone or in combination with 200 ppm of lysozyme on the microbial status, stability and physicochemical properties of the steamed sausage. Both compounds worked synergistically against lactic acid bacteria but no synergism was detected against total aerobes. Sensitivity of *L. moncytogenes* Scott A was found to be enhanced by other egg white proteins such as ovomucoid, conalbumin, and by alkaline pH conditions (Wang and Shelef, 1991).

Synergy with high hydrostatic pressure

High pressure processing (HPP) is one of the more extensively studied non-thermal preservation techniques that induce lethal or sublethal injury to bacteria with minimal effects on the organoleptic qualities of food. Several studies have looked into sensitization of Gram-negative bacteria to lysozyme under high hydrostatic pressure.

Hauben et al. (1996) observed the sensitization of *E. coli* MG1655 in phosphate buffer in the presence of 10µg/ml HEWL, using pressures in the range of 180 to 320 MPa. Cells were only sensitized to lysozyme during pressure exposure, because addition of lysozyme to pressurized cell suspensions immediately after pressure treatment did not cause any inactivation. Masschalck et al. (2001) studied the inactivation of six Gram-negative bacteria (*E. coli*, *Psuedomonas fluorescens*, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis*, *Shigella sonnei*, and *Shigella flexneri*) by high hydrostatic pressure treatment in the presence of hen egg-white lysozyme, partially or completely denatured lysozyme, or a synthetic cationic peptide derived from either hen egg white or colliphage T4 lysozyme. None of these compounds showed antibacterial effect at atmospheric pressure. But under high pressure, all organisms except both *Salmonella* species showed higher inactivation in the presence of 100µg of lysozyme/ml than without

this additive. They also found that complete removal of enzymatic activity of lysozyme by heat treatment fully eliminated its antibacterial properties under pressure, but partially denatured lysozyme was effective against some bacteria. The effect of high pressure homogenization on the activity of antimicrobial enzymes, lysozyme and lactoperoxidase against a selected group of Gram positive and Gram negative species inoculated in skim milk was studied by Vannini et al. (2004). The enzyme addition enhanced the efficacy of the pressure treatment and also affected recovery and growth of several of the tested species.

Incorporation into packaging films

Appendini and Hotchkiss (1997) investigated the feasibility of incorporating lysozyme into polymers which are suitable for food contact. Hen egg white lysozyme was immobilized on polyvinyl alcohol (PVOH) beads, nylon, 6, 6 pellets and cellulose triacetate (CTA) films and tested against a suspension of dried *Micrococcus lysoseikticus* cells. Polyvinyl alcohol and nylon, 6, 6 yielded low activity, while CTA yielded the highest activity. Padgett et al. (1998) studied the graduated levels of lysozyme (2.5, 5.0, 10, 17, 33, 66, and 133 mg/g of film) incorporated into cast films with corn zein against *L. plantarum* and various concentrations of lysozyme in combination with EDTA in corn zein cast films against *E. coli*. The minimum concentration of lysozyme that had some inhibition against *L. plantarum* was 10 mg of lysozyme/ g of film solution and maximum amount of lysozyme that could be incorporated without affecting physical properties of the film was 133 mg/g of film. Against *E. coli*, the maximum zone size was found with the combination of 66 mg lysozyme/ g of film and 30mM EDTA. The effect of whey protein isolate (WPI) films and coatings incorporating lysozyme on the inhibition of *L. monocytogenes* both in and on microbial media, as well as on cold-smoked salmon, were

studied by Min et al. (2005). WPI films incorporating 204 mg of lysozyme/ g of film (dry basis) inhibited the growth of a of 4.4 log cfu/cm^2 *L. monocytogenes* preparation. The WPI coatings incorporating lysozyme efficiently retarded the growth of *L. monocytogenes* at both 4 and 10°C. The anti-listerial effect of lysozyme-WPI coating was more noticeable when the coating was applied before inoculation than when the coating was applied after inoculation. Zein films incorporated with partially purified hen egg white lysozyme showed antimicrobial effect on *Bacillus subtilis* and *L. plantarum* (Mecitoğlu et al., 2006). By the addition of disodium EDTA, the films also became effective on *E. coli.* Conte et al. (2007) found that an immobilized lysozyme based active poly vinyl alcohol films were effective in inhibiting the growth of *M. lysodeikticus*.

IN-PACKAGE PASTEURIZATION OF RTE MEAT PRODUCTS

Effect on bacterial lethality

In-package pasteurization is an effective method to reduce post process surface contamination and can be an additional processing hurdle to reduce surface pathogen contamination. Cooksey et al. (1993) reported a 4 log reduction of *L. monocytogenes* in pre-cooked vacuum packaged beef using post-packaging pasteurization at 85°C for 16 min. The potential for *L. monocytogenes* to survive various times and temperatures of post-pasteurization in pre-cooked beef roasts was investigated by Hardin et al. (1993). They found that lethality of the treatment was directly related to an increase in dwell time and post pasteurization temperature. The impact of in-package pasteurization of Vienna sausages on survival of spoilage bacterial populations, primarily lactic acid bacteria (LAB) during storage at 8°C for 128 days was studied by Franz and von Holy (1996a). Depending on the severity of the applied thermal treatment, LAB were reduced from 84.4% of the total bacterial population to between 52.9 and 74.6% of the total population

for Vienna sausages. However, in-package pasteurization did not delay the rate of spoilage by eliminating these bacteria and also increased the predominance of *Bacillus* spp. Another study by the same authors (Franz and von Holy, 1996b) evaluated the heat resistance of three meat spoilage LAB (*Lactobacillus sake, Leuconostoc mesenteroides, Lactobacillus curvatus*). Decimal reduction values (D-values) at 57, 60, and 63°C were 52.9, 39.3 and 32.5 s for *L. sake*, 34.9, 31.3, and 20.2 s for *L. mesenteroides* and 22.5, 15.6, and 14.4 s for *L. curvatus,* respectively. The combination of organic acid addition and in-package pasteurization or pasteurization alone extended the microbiological shelf life (set to a level of 5×10^6 cfu/g) of Vienna sausages by 4-fold (2 week vs. 8week) compared to non-treated samples (Dykes et al., 1996). Roering et al. (1998) evaluated the effect of hot water pasteurization of vacuum-sealed packages of summer sausages on the survival of a three-strain mixture of *L. monocytogenes*. Bacterial numbers were reduced by about 3 log cfu /g within 30, 60, and 90 s at 99, 88, 77°C respectively, whereas numbers were reduced by < 2 log cfu /g after 240 s of heating at 66°C. The calculated D values were 2.08, 0.84, 0.37, and 0.28 min at 66, 77, 88, and 99°C, respectively. Murphy & Berrang (2002) found that post-process pasteurization of fully cooked vacuum packaged chicken breast strips in steam or hot water at 88 °C for 10 to 35 min lowered *Listeria innocua* population. There were 2 and 7 log reductions at 25 and 35 min, respectively. No significant difference was found on the survivors of *L. innocua* between steam and hot water treatments. Muriana et al. (2002) conducted a study on post package pasteurization of RTE deli meats by submersion heating for reduction of *L. monocytogenes*. A mixed cocktail of four strains of *L. monocytogenes* was resuspended in product purge and added to a variety of RTE meat products. Post-pasteurization at 90.6,

93.3, and 96.1°C for 2 to 10 min resulted in 2 to 4 log reductions in bacterial count. However, reductions of *L .monocytogenes* in product challenge studies were much lower than in the previous study to determine decimal reduction assays and the authors attributed this difference to a combination of surface imperfections that may shield bacteria from the heat and migration of chilled purge to the product surface. Ingham et al. (2005) conducted a study to evaluate small-scale hot-water post-packaging pasteurization as a post lethality treatment for *L. monocytogenes* on ready-to-eat beef snack sticks and natural-casing wieners. They obtained ≥ 2 log reductions in bacterial population when heated at 100°C for 1 min for individually packaged beef snack sticks and 4 min for packages of four sticks and seven sticks. A treatment of 7 min for packages of four natural –casing wieners achieved ≥ 1 log reduction in *L. monocytogenes*.

Heat resistance

Heat resistance of bacteria during in-package pasteurization is an important criterion for evaluating the effectiveness of in-package pasteurization. Heat resistance of the organism is usually expressed as Decimal reduction time (D- value) and z –value.

Murphy et al. (2003a) determined D and z values of *Salmonella* spp*, L. innocua, and L. monocytogenes* in different RTE poultry products during in-package pasteurization at different temperatures ranging from 55-70°C. Significant differences were found for the heat resistance of *Salmonella* spp*, L. innocua, and L. monocytogenes* among turkey, duck, and chicken products, indicating that the kinetic values of a certain pathogen in a specific product should be used for determining process lethality in fully cooked and vacuum-packaged poultry products during post-cook heat treatments. A study by McCormick et al. (2003) determined the D values of *L. monocytogenes* and *Salmonella* Typhimurium at various surface pasteurization temperatures for low fat turkey bologna

and demonstrated that complete inactivation of *S.* Typhimurium and *L .monocytogenes* cells can be achieved using an in-package pasteurization process. The D-values *for L. monocytogenes* at 61 and 65°C were 124 and 16.2 s respectively; whereas *S.* Typhimurium D-values were 278 s at 57 and 81 s at 60°C. z values were 4.44 and 5.56°C for *L. monocytogenes* and *S.* Typhimurium, respectively.

Factors affecting the efficiency

A limited number of studies have been conducted to determine the effect of product surface characteristics, product thickness, and packaging film thickness on the effectiveness of in-package pasteurization. The effect of thickness and composition on surface heating rate of bologna during in-package pasteurization was investigated by Mangalassary et al. (2004). Three thickness levels (4,12, and 20 mm) corresponding to 1, 3, and 5 slices bologna; two types of bologna having 13 and 18% fat content, and four pasteurization temperatures (60, 70, 80, and 90°C) were used in the study. Surface heating rate was fastest in the thinnest (4 mm) and slowest in the thickest (20mm) samples for all 4 temperatures. Surface heating rate was slower in bologna with the higher fat content compared with the lower fat bologna. Results from this study indicate that meat product thickness inside the bag and meat product fat content significantly affect surface heating rate and final surface temperature during in-package pasteurization of bologna and in turn the heat inactivation of pathogenic bacteria at the surface of the food product. The inactivation of *L. monocytogenes* during postcook in-package hot water pasteurization at 96°C was evaluated for fully cooked 4 kg turkey breast meat products by Murphy et al. (2003b). They found that the effectiveness of heat treatment for inactivating the pathogen was affected by product surface roughness. About 50 min of heating time was needed to achieve a thermal kill of 7 log cfu/cm^2 on products with

surface roughness up to 15 mm in depth. A study was conducted to determine the effect of packaging film thickness on thermal inactivation of *Salmonella and L. innocua* in chicken breast meat during hot water in-package pasteurization at 68°C for 10 to 120 s (Murphy et al., 2002). The results from this study indicated that increasing film thickness reduced heating rate and subsequently reduced thermal inactivation for the organisms. More than a 2 log difference in reduction of *Salmonella and L. innocua* was reported between the meat packaged in 0.0762 and 0.2032 mm thick film. Murphy et al. (2003c) suggested a model to predict thermal lethality of *Salmonella* spp. and *L. innocua* for different thicknesses of fully cooked, vacuum packaged chicken breast meat products during post cooking in-package pasteurization. This model can be used to predict the heat treatment time needed to achieve 7 log reductions of *Salmonella* spp. or *L. innocua* for different thicknesses of fully cooked vacuum-packaged chicken breast meat products that would be pasteurized in a hot-water cooker at a treatment temperature of 90°C. The model prediction was validated with an inoculation study involving the same product at a 95% confidence level up to 10^7cfu/g for *Salmonella* spp. *and L. innocua*.

Combination with antimicrobials

Combining in-package pasteurization with antimicrobial agents is a novel approach in food preservation. This approach may help to reduce the heat resistance of the organism and thereby to reduce the duration of severe heat treatment in the form of in-package pasteurization. Murphy et al. (2004) studied the effect of sodium lactate on thermal inactivation of *L. monocytogenes* and *Salmonella* spp. in ground chicken thigh and leg meat during in-package pasteurization at temperatures ranging from 55-70°C. No significant difference was found for the D-values of *Salmonella* spp. at 55 to 70°C between the meat with and that without sodium lactate. The z values of both *L.*

monocytogenes and Salmonella spp. were not affected by sodium lactate. Chen et al. (2004) combined surface application of pediocin (ALTA 2341) with post-packaging thermal pasteurization in hot water at 71, 81, or 96°C for 30, 60, or 120 s for control of *L. monocytogenes* on frankfurters. *L. monocytogenes* was reduced by all treatments, but 81°C or higher temperatures for at least 60 s in combination with pediocin were necessary to achieve at least a 50% reduction of initial populations. Little or no growth occurred on frankfurters for 12 weeks at 4 or 10°C, and for 12 days at 25 °C. The inhibitory effects of in-package pasteurization combined with a nisin containing wheat gluten film were tested over 8 weeks storage period against *L. monocytogenes and S.* Typhimurium populations inoculated on refrigerated bologna (McCormick et al., 2005). Bologna slices subjected to in-package pasteurization process reduced *L. monocytogenes* populations 3.8-7.0 log cfu/g and the remaining population fluctuated between 1.2-3.8 log cfu/g over 2 months refrigerated storage period. *S*. Typhimurium was reduced 5.7-7.3 log cfu/g and the remaining population progressively declined from 100 to <10 cfu/g over 2 months storage. Combining both treatments significantly reduced *L. monocytogenes* populations and prevented outgrowth over 2 months storage period but provided no added inhibitory effect against *S*. Typhimurium compared with only pasteurization.

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CHAPTER 3
THICKNESS AND COMPOSITIONAL EFFECTS ON SURFACE HEATING RATE OF BOLOGNA DURING IN-PACKAGE PASTEURIZATION

ABSTRACT

The surface heating rate (γ) and final surface temperature (α) during in-package pasteurization were determined for different thickness levels of 2 types of bologna having different (13 and 18%) fat contents. Three different thicknesses (4, 12, and 20 mm) corresponding to 1, 3, and 5 slices of bologna, were vacuum-packaged separately in a clear polymer pouch after placing thermocouple on the surface. Refrigerated samples were immersed in a water bath set to 1 of 4 predetermined temperatures (60, 70, 80, and 90° C), and time and temperature data were recorded for 10 min. Surface- heating rate was fastest in the thinnest (4mm) and slowest in the thickest (20mm) samples for all 4 temperatures. Surface- heating rate was slower in bologna with the higher fat content compared with the lower fat bologna. Final surface temperature attained after 3 min was lower with increased thickness levels for all temperatures. Thus, meat sample thickness and fat content significantly affect surface heating rate and final surface temperature during in-package pasteurization of bologna.

INTRODUCTION

Ensuring the safety of meat and meat products is always a challenge to researchers. Reports of microbial foodborne illness and contaminated product recalls have been on the increase in recent years. Information obtained from Food Safety and Inspection Service records indicates that, in 2002, there were 81 recalls of meat and poultry products resulting from microbial contamination alone, accounting for 56.78 million pounds

(FSIS, 2002). About half of these recalls were for ready-to-eat (RTE) meat products. Contamination of RTE meats with *Listeria monocytogenes* and *Salmonella* spp. occurs mainly at postprocessing (Mbandi and Shelef, 2002).

Numerous food preservation methods including heating, chilling, freezing, curing, smoking, acidification, and irradiation can be used to inhibit microbiological spoilage and to prevent foodborne disease (Leistner, 1987). Thermal processing is considered one of the most efficient methods and various techniques have been developed to thermally process food before and after packaging (Farkas, 1997). Postprocess handling is a cause of recontamination of RTE meat products. In-package pasteurization is used as an effective method to reduce postprocess surface contamination and can be an additional processing hurdle to reduce surface pathogen contamination. Cooksey et al. (1993) reported reduction of *L. monocytogenes* in precooked vacuum-packaged beef using postpackaging pasteurization in 85°C water for 16 min. The potential for *L. monocytogenes* to survive various times and temperatures of postpasteurization in precooked beef roasts was investigated by Hardin et al. (1993). They found that lethality of the treatment was directly related to an increase in dwell time and postpasteurization temperature. Murphy and Berrang (2002) found that postprocess pasteurization of fully cooked vacuum-packaged chicken breast strips with hot water or steam lowered *L. innocua* population.

Muriana et al. (2002) conducted a study on postpackage pasteurization of *L. monocytogenes*-inoculated RTE deli meats and achieved a 2 to 4 log cycle reduction when processed at 90.6, 93.3, or 96.1°C and heated for 2 to10min.

A study by McCormick et al. (2003) demonstrated that significant reductions in bacterial populations and complete inactivation of *Salmonella* Typhimurium and *L. monocytogenes* could be achieved using an in-package thermal pasteurization process. Murphy et al. (2003) suggested a model to predict thermal lethality of Salmo*nella and L. innocua* for different thicknesses of fully cooked, vacuum-packaged chicken breast meat products during post-cooking in-package pasteurization. Commercial in-package pasteurization systems for meat have determined that several minutes of pasteurization is required to obtain 2 to 3 log reductions in target bacteria (Unitherm, 2003). Meat thickness during in-package pasteurization may have an effect on the surface temperature and therefore, on the efficiency of the pasteurization. Although numerous studies report on the effect of meat thickness and composition on heat penetration, there are limited studies on their effect on surface heating rates. The objective of this study was to evaluate the effect of product thickness on the surface heating rate and final surface temperature during in-package pasteurization for 2 bologna types differing in fat content.

MATERIALS AND METHODS

Product preparation

Two types of RTE meat bolognas were used in this experiment; a beef bologna containing 12 g (18%) of fat and 5 g of protein per serving, and a mixed species type containing chicken, pork, and beef with 8 g (13%) of fat and 3 g of protein per serving. Bologna slices with a thickness of 4 mm were cut into 16-cm^2 pieces using a sterile cutting template and the pieces were stacked together as in a commercial package to achieve the desired thickness level.

Three thicknesses (4, 12, and 20 mm) corresponding to 1, 3, and 5 slices of bologna were used in this study. K-type Teflon insulated thermocouples (Omega Engineering Inc., Stamford, CT) were placed on the surface of the samples. Using forceps, each of the samples (thickness levels) was separately placed inside a clear polymer pouch (Model P64x, Cryovac, Duncan, SC). The pouch is conventionally laminated with a nylon skin, a linear low-density polyethylene sealant, and a nylon barrier film. The pouch had an oxygen transmission rate of 60 mL/m^2 per day at 23°C and 0% RH. Pouches were sealed using a vacuum sealer (Koch Model UV 250, Koch Supplies Inc., Kansas City, MO) and held at 4 ± 2°C overnight.

Thermal processing (Pasteurization)

Thermal Processing (Pasteurization) Thermal processing of bologna was performed using a Precision digital water bath (Microprocessor controlled 280 series water bath, Jouan Inc., Winchester, VA) set to 1 of 4 predetermined temperatures (60, 70, 80, or 90°C). Refrigerated samples were directly immersed into the water bath. Time and temperature data were recorded using a channel datalogger (CALPlex 32, TechniCAL, New Orleans, LA) and thermal processing software (CALSoft Version 1.32, TechniCAL, New Orleans, LA) for10 min.

Experimental Treatments

Three thickness levels, 2 types of bologna, and 4 pasteurization temperatures were used in this study. In a single experiment, 3 packages containing 3 different thicknesses [4 mm (1 slice), 12 mm (3 slices), and 20 mm (5 slices)] of the same type of bologna were immersed in the water bath at one of the specific pasteurization temperatures. The experiment was replicated 3 times on different days using 3 separate batches of meat.

Heating rate models

Two models were considered for calculating surface heating rate in this study. The first was a double exponential that is often used to describe the relationship between temperature and time as in this study. For some exponential decays, where the decline toward asymptotic value appears prolonged, with a decay rate in later stages that is slower than would be expected, a double exponential formation may be necessary. This model has the form of

$$y_i = \alpha + \beta_1 \exp(-\lambda_1 x) + \beta_2 \exp(-\lambda_2 x) + e_i$$

where y_i is the surface temperature of replicate i, x is the time of pasteurization, α is the surface temperature of the asymptote as x reaches ∞, and $\alpha + \beta_1 + \beta_2$ is the surface temperature at x = 0. The rate of increase is given by

$$-\lambda_1\beta_1 \exp(-\lambda_1 x) - \lambda_2\beta_2 \exp(-\lambda_2)$$

where e_i is the random error for replicate i. Estimates for the parameters α, β_1, λ_1, β_2,and λ_2 were obtained using the NLIN procedure from SAS (Version 8.0, SAS Institute, 2000). Although the model fit well for many of the replicates, there were 2 problems. First, the NLIN procedure failed to converge for several replicates, and secondly, there were cases when the estimates for λ_1 varied greatly for different replicates (from values of 10^{-1} to 10^{10}). This suggested the estimates may not follow the properties of the samples and that is the model may be "over fitting" the data.

In this study, 2 asymptotes are not parallel, but perpendicular. This is considered a special case of the exponential form, which yields a rectangular hyperbola. Therefore a rectangular hyperbola model was considered next for this experiment because the value on the y axis (surface temperature) starts at about 4°C in all thickness levels during in-

package pasteurization and increases to a maximum plateau value (set pasteurization temperature), over time.

A rectangular hyperbolae model was considered having the form of

$$y_i = \alpha + \beta/(1 + \gamma x) + e_i$$

where y_i is the surface temperature for replicate i, x is the time, γ is the heating rate, α is the asymptote as x reaches ∞, $\alpha + \beta$ is the surface temperature at $x = 0$, and e_i is the random error for replicate i. The rate of increase is given by $\gamma\beta/(1 + \gamma x)^2$.

When observations are obtained at $x > 0$, this form closely mimics the exponential form:

$$y_i = \alpha + \beta \exp(-\lambda x).$$

Estimates for the parameters α, β, and γ were obtained using the NLIN procedure from SAS (Version 8.0, SAS Institute, 2000). Although in many cases this model did not fit as closely as the double exponential, it did converge for all replicates and the parameter estimates did not vary to the same extent as with the double exponential model. It was felt that the rectangular hyperbolae model better fitted the physical phenomenon than the double exponential model. Results applied to the second model were used for further analyses.

Statistical Analysis

The effects of bologna thickness (4, 12, and 20 mm), bologna type (beef and mixed species) and water bath temperature (60, 70, 80, and 90°C) on bologna surface heating rate and final surface temperature during in-package pasteurization were evaluated. Specifically, the parameters square root of γ (surface heating rate) and α (final surface temperature) from the rectangular hyperbolae previously described were statistically

analyzed. The 3 treatment factors were arranged in a 2 × 4 × 3 complete factorial design. A split-plot design was used with bologna type and water bath temperature as the whole plot factors in a completely randomized design and bologna thickness as the subplot factor. Fisher's protected least significant difference was used for all multiple comparisons. The analyses were carried out using the mixed procedure of SAS (Version 8.0, SAS Institute, 2000).

RESULTS AND DISCUSSION

Surface heating rate (γ)

Because the estimates for γ were not normally distributed, a square root transformation was used, resulting in a normal distribution for surface heating rates. The surface heating rate was slower with increased thickness levels (Figure 3.1, lower fat bologna) and slower in bologna with higher fat content (Figure 3.2). There was a difference ($P < 0.0001$) in the mean value for square root of γ for 3 thickness levels at all 4 pasteurization temperatures used in the study with the thinnest samples having the fastest rate of surface heating (Table 3.1). This difference in surface heating rate between different thickness levels may be due to a heat sink effect. During pasteurization, heat from the water bath passes through the package to the bologna surface and diffuses to the meat interior. The thicker the meat sample, the greater the diffusion of heat energy to the meat interior, resulting in a loss of surface heat energy. After pulling heat away from the meat surface, the meat bulk accumulates heat energy. The heat slowly diffuses back to the surface until the interior and surface reach the final set temperature. Considering this phenomenon, thickness of meat and meat product composition are important factors in attaining the required surface temperature. As meat thickness increases, surface heating

rate decreases. Therefore, product thickness is an important factor for in-package surface pasteurization of meat and meat products. For uncooked meat products, the cold spot at the food core is the most important consideration for thermal processing. However, with RTE meats, post-processing contamination is a surface phenomenon, making the meat surface the main concern of the thermal treatment. Furthermore, determination of a mathematical model to predict the relationship between meat thickness and surface heating is important in developing surface pasteurization processes.

Murphy et al. (2002) reported that packaging film thickness has an effect on heating rate during in-package pasteurization. In contrast with our results, they expressed the view that the effect of product thickness would be more important for internal pasteurization of packaged meat or poultry products. Previous studies found that in-package pasteurization results in sufficient log reductions of surface bacterial populations (Muriana et al., 2002; McCormick et al., 2003). Thus, attaining a rapid surface heating rate would be of great importance in reducing the pasteurization time and maintaining the quality of the product.

Difference ($P < 0.0001$) was also found for square root of γ for the 2 bologna types. The mixed species bologna with a lower fat content had a faster surface heating rate compared with the higher fat beef bologna. These results were same for the 4 pasteurization temperatures used in the study (Table 3.1). The lower heating rate in high fat bologna could be attributed to the difference in heat transfer mechanism. The decreased surface heating rate in bologna with higher fat content may be due to the insulating effect of fat to heat. Woodams and Nowrey (1968) stated that an increase in fat content resulted in a decrease in thermal conductivity of meat products because the

thermal conductivity of fat is less than that of lean meat. However, Shilton et al. (2002) observed that heat transfer in higher fat beef patties during cooking did not occur by conduction alone, but also by internal convection resulting from movement of moisture and fat, whereas in low fat beef patties, heat transfer was thought to occur by conduction only. Thus, the 2 modes of heat transfer (conduction and convection) may have reduced the surface heating rate in meat with higher fat content. Although one bologna was from mixed species (lower fat) and the other was from pure beef (higher fat), proximate composition of a comminuted product has a strong effect on thermal conductivity, particularly moisture content followed by fat and protein. A formula estimating the specific heat of food was proposed by Singh and Heldman (1984) using the mass of the proximate components (carbohydrate, protein, fat, ash, and water). Despite thermal conductivity models that support this, the authors cannot overlook the possibility that the different meat species sources did not affect surface heating to some extent. It can be stated that these 2 types of bologna products differed in surface heating rate. Both the conduction and convection of heat from the meat surface and transfer of heat throughout the product may have an effect on surface heating rate. There was no difference (P = 0.2524) in the mean value for square root of γ for the set pasteurization temperatures. There was no interaction ($P > 0.05$) between any of these 3 factors (thickness, bologna type, and pasteurization temperature).

Final Surface Temperature (α)

From a practical standpoint, federal regulations require that an endpoint temperature be reached in cooking raw meat products. The same strategy could be applied to ensure the safety of packaged RTE meat products. Temperature is easier to measure and record

in the processing environment than calculation of a log reduction. Thus, various factors such as meat thickness and meat composition can be compared for their effect on heating rate by measuring the final temperature attained after a specific time.

The meat surface temperature (α) attained after 10 min of pasteurization was lower with increased thickness levels (data not shown). There was a difference ($P < 0.0001$) in the mean value of α for 3 thickness levels at 4 pasteurization temperatures. The higher final surface temperature attained in the thinnest sample resulted from a faster surface heating rate compared with thicker meat samples. This trend was evident even for a shorter pasteurization time of 3 min (Table 3.2). The difference in 3-min bologna surface temperature between 4 and 12 mm thick samples ranged from 7.2 to 9.3°C for 18% fat samples for the 4 pasteurization temperatures. The 3 min surface temperature for 13% fat bologna was also constant across the 4 pasteurization temperatures, and ranged from 4.6 to 6.3°C. The thinner sample/faster heating rate trend was less evident when the 12-and 20-mm samples were compared, ranging from 1.6 to 5°C (18% fat) and 3.5 to 5.5°C (13% fat). Thus, thickness of the product plays an important role in attaining the required surface temperature by a set pasteurization time, which is essential in reducing the surface pathogen contamination.

The impact of surface heating rate on microbiological safety can be estimated by using the experimentally measured D-and z-values for specific product conditions. Using the D-and z-values determined for in-packaged pasteurized bologna, the process time required to attain a 5 log reduction in *L. monocytogenes* can be calculated. The impact of sample thickness on attaining a surface 5 log reduction at various temperatures was 2.5 to

6 times longer for 20 mm thick samples compared with 4 mm thick lower fat samples (Table 3.3). A similar trend was found for the higher fat (18%) bologna.

CONCLUSIONS

Rapid processing can reduce costs and increase efficiency, therefore product thickness has a significant effect on in-package pasteurization of RTE meats. The USDA issued a rule in October 2003 that certain RTE meat and poultry products must implement 1 of 3 risk-based alternatives with written program and verification through testing to control *L. monocytogenes*. A post-lethality treatment is included in 2 of the 3 alternatives. A practical approach of in-package pasteurization as a post-lethality treatment to target surface *L. monocytogenes* would reduce the risk of its presence. Thus, factors affecting the surface heating rate, such as product thickness, need to be clearly understood to eliminate the pathogen and its related costs to the industry.

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Table 3.1. Surface heating rates (√°C/s) for 3 thicknesses of 2 types of bologna during in-package pasteurization.

	18% fat			13% fat		
	Thickness			Thickness		
Processing Temperature (°C)	4mm	12mm	20mm	4mm	12 mm	20 mm
60	0.277[b]	0.153[d]	0.155[f]	0.347[a]	0.247[c]	0.177[e]
70	0.259[b]	0.239[d]	0.180[f]	0.372[a]	0.248[c]	0.234[e]
80	0.303[b]	0.199[d]	0.168[f]	0.349[a]	0.239[c]	0.198[e]
90	0.268[b]	0.216[d]	0.154[f]	0.348[a]	0.229[c]	0.187[e]

[a-e]Means within a row lacking a common superscript differ ($P < 0.0001$)

Table 3.2. Surface temperature (°C) at 3min for 3 thicknesses (4, 12, 20 mm) of 2 types (13 and 18% fat) of packaged bologna during in-package pasteurization.

	18% fat			13% fat		
	Thickness			Thickness		
Processing Temperature (°C)	4mm	12mm	20mm	4mm	12 mm	20 mm
60	59.40[a]	50.15[b]	48.51[c]	59.62[a]	53.25[b]	49.71[e]
70	69.36[a]	62.15[b]	57.35[c]	69.76[a]	65.11[b]	61.72[e]
80	79.12[a]	70.89[b]	65.78[c]	80.03[a]	74.82[b]	69.29[e]
90	89.00[a]	79.97[b]	72.99[c]	89.42[a]	83.26[b]	77.21[e]

[a-c]Means within a row and for each meat type (%fat) lacking a common superscript differ ($P < 0.0001$).

Table 3.3. Calculated time[1] (min) required to achieve 5 log reductions in *Listeria monocytogenes* population for 3 thicknesses (4, 12, and 20mm) of packaged bologna (13% fat) during in-package pasteurization at different temperatures.

	Pasteurization temperature (°C)			
Thickness (mm)	60	70	80	90
4	>10	1.5	0.72	0.35
12	>10	5.4	2.56	0.59
20	>10	9.5	4.12	0.97

[1]The 5 log reduction was calculated based on D- and z- values determined from in-package pasteurized bologna by McCormick et al. (2003).

Fig. 3.1. Surface temperature profile for 3 thicknesses of packaged bologna (13% fat) corresponding to 1 (4mm), 3 (12mm), and 5 (20mm) slices during in-package pasteurization at 60°C.

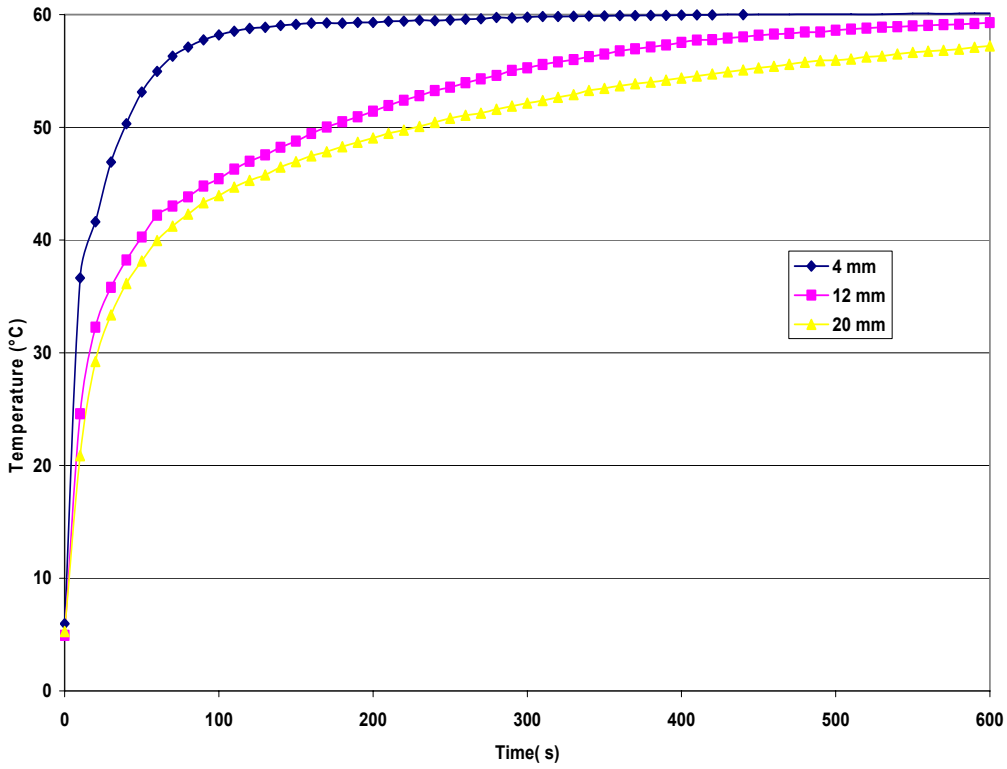
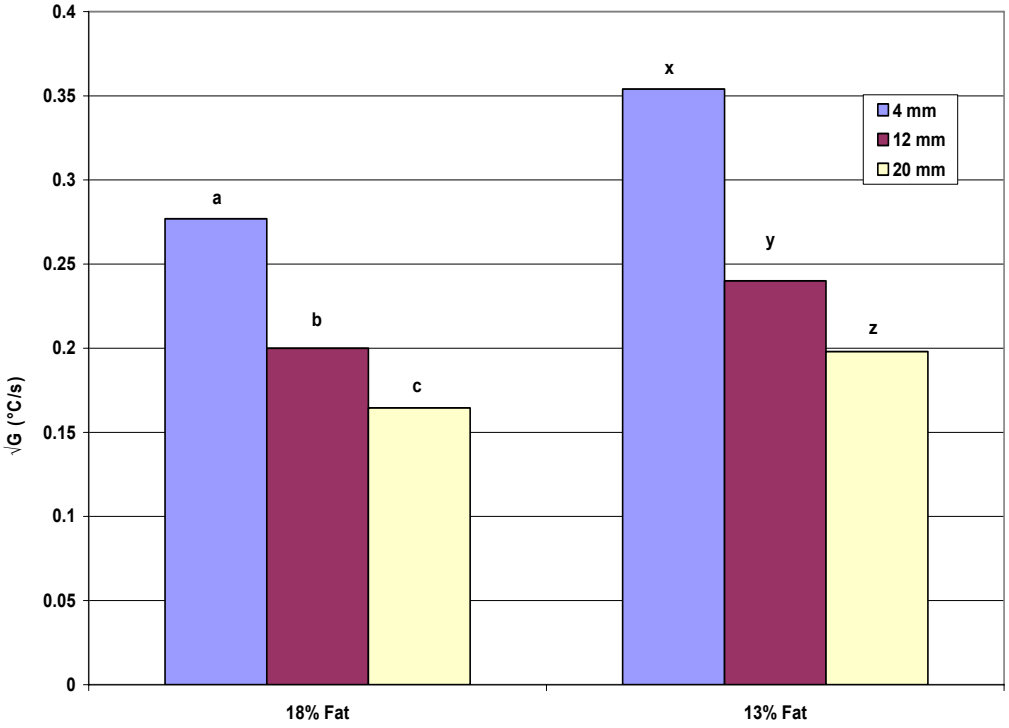


Fig. 3.2. Surface heating rates for 3 thicknesses of 2 types of packaged bologna during in-package pasteurization for 4 temperatures pooled. [a-c;x-z]Values within fat content marked with different letters differ at $P < 0.0001$.



CHAPTER 4
EFEECT OF COMBINING NISIN AND/OR LYSOZYME WITH IN-PACKAGE PASTEURIZATION ON THERMAL INACTIVATION OF *LISTERIA MONOCYTOGENES* IN READY-TO-EAT TURKEY BOLOGNA

ABSTRACT

Achieving the targeted lethality with minimum exposure to heat is a challenge as well as a need to the poultry industry to preserve the product quality during pasteurization. The objective of this study was to evaluate the effect of surface application of nisin and/or lysozyme in combination with in-package pasteurization of ready-to-eat (RTE) low fat turkey bologna on the inactivation of *L. monocytogenes*. Sterile bologna samples were treated with solutions of nisin (2mg/ml = 5000 AU/ml), Lysozyme (10mg/ml = 80 AU/ml), and a mixture of nisin and lysozyme (2mg nisin + 10 mg lysozyme/ml). Bologna surfaces were uniformly inoculated with a *Listeria* suspension resulting in a population of 8 log cfu/slice. Samples were vacuum-packaged and subjected to heat treatment (60, 62.5 or 65°C). Two non-linear models (Weibull and log-logistic) were used to fit the data and using the parameters of the models, time needed to achieve a 4 log reduction was calculated. Nisin-lysozyme combination and nisin treatments were effective in reducing the time required for 4 log reductions at 62.5 and 65°C, but not at 60°C. At 62.5°C, nisin-lysozyme treatment required 23% less time than the control sample to achieve 4 log reductions and 31% less time at 65°C. Lysozyme alone did not show enhanced antilisterial activity with heat. Results from this study can be useful to the industry in developing an efficient intervention strategy against contamination of RTE meat products by *L. monocytogenes*.

INTRODUCTION

Post-cooking contamination of RTE meat and poultry products by *L. monocytogenes* is a major food safety problem as well as an economic hardship to the food industry. *L. monocytogenes* is an important foodborne pathogen that can cause life-threatening invasive infections in neonates, pregnant women, elderly, and immunocompromised individuals (Slutsker and Schuchat, 1999; Gerba et al., 1996). In 2002, a major outbreak of listeriosis in northeastern United States associated with sliced turkey deli meat resulted in 46 cases including 7 deaths (CDC, 2002). Majority of the food product recalls associated with listeriosis involve RTE meat and poultry products. These food product recalls have serious economic and public perception impacts on the food industry (Ivanek et al., 2004; Kramer et al., 2005; Marsh et al., 2004; Teratanavat and Hooker, 2004; Thomas and McKenzie, 2001).

Post-process operations in the preparation of RTE meat products such as peeling, sorting, loading, and slicing are potential sources for recontamination of the products with *L. monocytogenes* (Murphy et al., 2005). The organism can survive in food-processing facilities for long periods (Tompkin, 2002) and thus, processing equipment and other food contact surfaces can act as a source of this organism during post-process operations. In deli meat products, contaminated slicers act as an important contamination site of the organism (Humphrey, 1990; Hudson and Mott, 1993). Because of the ability of *L. monocytogenes* to grow and multiply at refrigeration temperatures in vacuum packaged meat products, even a low level of initial contamination could result into a substantial number by the time of product consumption (Seman et al., 2002). The fact that most RTE

and deli meat products are often consumed without further heating adds to the seriousness of the problem.

Because of the high fatality rate of listeriosis in human beings, and also due to the high susceptibility of RTE meat products to *Listeria* contamination and growth, the United States Department of Agriculture (USDA) implemented a "zero-tolerance" policy for this pathogen in RTE products (USDA, 1989). In 2003, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) released an interim final rule to control *L. monocytogenes* for post-lethality treatments used for RTE meat and poultry products (USDA-FSIS, 2003). This ruling includes three alternative approaches that establishments can implement in the processing of RTE meat and poultry products using a post-lethality treatment, antimicrobial agent, and sanitation control measures.

Various post-lethality treatments such as surface pasteurization, high pressure processing, pulsed electric field, irradiation, and use of antimicrobial agents have been tested to reduce the contamination and to eliminate *L. monocytogenes* from RTE meat and poultry products (Zhu et al., 2005; Luchansky et al., 2006). Thermal processing is one of the most efficient methods in controlling microbial contamination of food products. Heat treatment in the form of pre and post-package pasteurization using hot water or steam was found to be an effective method to reduce post-process surface contamination by *L. monocytogenes* (Muriana et al., 2004; Murphy et al., 2003; McCormick et al., 2003; Roering et al., 1998). Murphy and Berrang (2002) found that post-process pasteurization of fully cooked vacuum packaged chicken breast strips in steam or hot water at 88°C for 35 min resulted in 7 log reduction in *Listeria innocua* population. Effect of various factors like product thickness, composition, product surface

characteristics, and packaging film thickness on the efficiency of in-package pasteurization have also been investigated (Mangalassary et al., 2004; Murphy et al., 2003; Murphy et al., 2002). Researchers found that various product characteristics have an effect on the total bacterial lethality during in-package pasteurization. Another important factor is the time or duration of pasteurization since processors would prefer shorter treatments which would be cost effective and less detrimental to product quality. Combining in-package pasteurization with other preservative methods may be an effective approach to reduce pasteurization times in attaining a targeted bacterial lethality.

Surface application of various antimicrobial agents alone as well as in combination are also effective at controlling *L. monocytogenes* in RTE meats (Gill and Holley, 2000; Samelis et al., 2001). Post-process surface application of antimicrobials may be more advantageous than addition to the formulation because the cells may be present on the surface and also surface application in smaller quantities may have less potential for negative effects on the sensory qualities of the product (Samelis et al., 2001; Chen et al., 2004).

Nisin and lysozyme are two antimicrobials approved for many food applications. Nisin is an antibacterial peptide produced by *Lactococcus lactis* subsp. *lactis* that effectively inhibits Gram-positive bacteria (Delves-Broughton et al., 1996). Nisin is one of the most commonly used natural antimicrobial compounds and was approved for use in food in 1969 and was awarded generally recognized as safe (GRAS) status in the United States in 1988 (FDA, 1988). Nisin has shown synergistic antibacterial activity with heat against *L. monocytogenes* (Knight et al., 1999; Budu-Amako et al., 1999; Modi

et al., 2000). This synergism may yield targeted lethality in shorter time when nisin is combined with in-package surface pasteurization. Lysozyme is an enzyme found in biological fluids and tissues, which lyse the cell walls of certain Gram-positive bacteria by splitting the bond between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in the bacterial cell wall (Proctor and Cunningham, 1988). Enhanced antibacterial activity of a mixture of nisin and lysozyme against pathogenic and spoilage bacteria was reported by various investigators (Chung and Hancock, 2000; Nattress and Baker, 2003; Gill and Holley, 2000). But studies on synergy of this mixture along with heat have not been reported.

Considering the synergy of the various intervention methods explained in previous studies mentioned above, combining in-package pasteurization along with pre-surface application of nisin and lysozyme may have a positive effect on achieving the targeted bacterial reduction with shorter pasteurization treatment. Therefore the objective of this study was to evaluate the surface application of nisin and/or lysozyme in combination with in-package pasteurization of RTE low fat turkey bologna to eliminate *L. monocytogenes*, particularly to see the changes in heat inactivation kinetics of the organism.

MATERIALS AND METHODS

Food product preparation

Ready-to-eat low fat turkey bologna averaging 14.3% fat, 10.7% protein, and 71.4% moisture was used for the experiment. Bologna samples were batch irradiated for 521 min using a cobalt 60 source with a total dose of 2.4 Mrad at 4,607 R/minute at Auburn University. Irradiation was carried out to eliminate background flora before inoculation

studies. Bologna samples were kept frozen at -70°C and thawed overnight at 4°C prior to experimentation. For inoculation and thermal inactivation studies, bologna slices of approximately 2.5 mm thickness were cut into 4cm × 4cm pieces using a sterile cutting template and each piece was used as an experimental unit.

Culture preparation

L. monocytogenes ATCC 15313 was preserved by freezing the cultures at -70°C in vials containing brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 20% (v/v) glycerol (Sigma, St. Louis, MO). To propagate the culture, a frozen vial was thawed at room temperature, and 0.1 ml of the thawed culture was transferred to 9.9 ml of BHI broth in screw-cap tubes and incubated aerobically for 16-18 h at 37°C with agitation at 200 revolutions per min (Thermolyne Maxi-Mix III type 65800, Barnstead/Thermolyne, Dubuque, IA). The inoculum was prepared from a second transfer of this culture (0.1ml) to another 9.9 ml tube of BHI broth, and incubated aerobically for 16-18 h at 37°C with agitation. After incubating for 16 h, a washed cell suspension of the organism was prepared by harvesting the cells by centrifugation at 3000 × g (IEC HN-SII centrifuge, International Equipment Co., Inc., Needham Heights, MA), washing with 10 ml of 0.1% sterile peptone water (Bacto peptone, Difco Laboratories, Detroit, MI), and resuspending in 0.1% sterile peptone water to obtain a population of approximately 8- 9 $\log_{10}$ cfu/ml. Initial cell populations were verified by enumerating the cells after pour-plating in BHI agar and incubating at 37°C for 48 h.

Antimicrobial preparation

Nisin

Nisaplin, a commercial nisin product (2.5% nisin with 10^6 IU/g) was provided by Danisco (Danisco USA Inc, New Century, KS). Solutions of required concentrations of nisin were prepared on the day of the experiment by dissolving appropriate amount of nisin in sterile distilled water.

Lysozyme

Egg white lysozyme was provided by Q.P. Corporation (Q.P. Corporation, Japan). Solutions of lysozyme were prepared on the day of the experiment by dissolving appropriate amount in sterile distilled water.

Antimicrobial activity assay

Activities of nisin and lysozyme were determined by critical dilution assay (Pucci et al., 1988). Serial two-fold dilutions of the antimicrobial agents were tested against *L. monocytogenes* ATCC 15313. Ten µl of each dilution was spotted on the surface of the BHI agar medium seeded uniformly with a suspension of *L. monocytogenes*. After incubation (48h at 37°C), the plates were checked for zones of inhibition. Titer of nisin and lysozyme in Arbitrary Units per ml (AU/ml) was expressed as the reciprocal of the highest dilution showing a definite inhibition zone. Activity of the antimicrobial compounds was expressed in AU/mg based on the weight of the antimicrobial compounds used in serial dilution and converted into AU/ml based on the weight (mg) of the antimicrobial compound used in the application solution.

Antimicrobial treatment and inoculation

Sterile, frozen bologna samples were thawed overnight at refrigeration temperature (4±1°C). On the day of the experiment, bologna samples were removed from refrigerator and aseptically transferred to a sterile surface under a Germfree ®Bioflow chamber (Ormond Beach, FL). Each bologna slice was cut into 4cm × 4cm pieces using a sterile cutting template as previously described. Meat samples for the thermal treatment were uniformly surface-treated with 0.1 ml of the antimicrobial solutions. The activity units applied for each treatment were control: 0, nisin: 500 AU (5000 AU/ml=2 mg/ml), lysozyme: 8 AU (80 AU/ml=10 mg/ml), and nisin-lysozyme: 508 AU (5000 AU nisin + 80 AU lysozyme/ml). After the antimicrobial treatment, all bologna surfaces were inoculated uniformly with a 0.1 ml of a 9 log cfu/ml suspension of *L. monocytogenes* to have a cell concentration of 8 log cfu/slice. All samples were aseptically transferred to a post-pasteurization bag (Model CNP-310, Cryovac, Duncan, SC). Insulated thermocouples (K type Teflon, Omega Engineering, Inc, Stamford, CT) were attached to the surface of two un-inoculated bologna samples to measure the temperature during thermal treatment. All bags were then vacuum packaged (Koch Model, UV 250, Koch Supplies Inc, Kansas City, MO) (vacuum: 98, seal: 2, and gas: no)

Thermal processing and heat resistance determination

Packaged bologna samples were simultaneously submerged into a water bath (Precision, model 186, Precision Scientific Incorporated, Chicago, USA) set to a pre-determined temperature (60, 62.5 and 65°C). The surface temperature and time data were monitored using a channel datalogger (CALPlex 32, TechniCAL, New Orleans, LA) and thermal processing software (CALSoft, Version 1.32, TechniCAL, New Orleans, LA)

during the entire process. Once the temperature of bologna surface reached the equilibrium temperature, samples from each treatment were removed from the water bath at selected time intervals (Total 7 samples in each run with 10, 20, and 120 s time interval for 65, 62.5, and 60°C, respectively) and rapidly cooled by immersing in ice-water slurry for 10 s to minimize any further heat effect. Each sample was subsequently aseptically removed from the pasteurization bag and homogenized with 99 ml of sterile 0.1% peptone water by placing the samples into a stomacher (Seward Stomacher 400 Circulator, Seward, Inc, UK) for 1 min at 230 rpm. Homogenates were then serially diluted and appropriate serial dilutions were pour plated in duplicate using BHI agar. Plates were incubated at 37°C for 48 h before enumerating colonies. Colony forming units (cfu) counts were converted to $\log_{10}$ cfu/cm^2 of the sample for the analysis of the data.

One set of all 4 treatments were sampled microbiologically without any heat treatment to enumerate survivors at 0 time.

Statistical Analysis

Experiment was replicated 3 times at each pasteurization temperature.

Initial inhibitory effect of antimicrobial agents

A 2 × 2 factorial design was used to statistically analyze the initial inhibitory effect of antimicrobial agents at 0 h before in-package pasteurization. Two levels of nisin and lysozyme (present, not present) and the interaction of nisin*lysozyme was used in the model. Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2003). Least square difference (LSD) multiple comparison procedure was used to evaluate significant differences ($P < 0.05$) among means.

Bacterial lethality for antimicrobial-heat treatment combination

Log reductions were calculated by subtracting the log count for each treatment after specific duration of heating from the initial log count obtained for the untreated control sample before heating. Treatment differences ($P < 0.05$) within each pasteurization temperature were evaluated using LSD multiple comparison procedure.

Modeling of inactivation curves

Survivor log counts obtained for all treatments over time were tested for linearity using orthogonal polynomials. None of the survivor "curves" tested was linear. Therefore various non-linear mathematical models were tested to obtain a "best fit" to the data. A single model could not be satisfactorily used to fit all survivor curves at the 3 different temperatures. Therefore both a Weibull model and a modified log-logistic model were selected to model the data for all treatments and temperatures. The goodness of fit by the two models was determined using an extra sum of squares test (Ramsey and Schafer, 2002). The model having the better fit for each treatment at a particular temperature was selected and used from that point forward.

Weibull model

The cumulative form of the Weibull distribution suggested by Peleg & Cole (1998) was used to describe survivor curves obtained at 62.5 and 65°C. Its equation is given by

$$\text{Log}\ \frac{N}{N_0} = -\,bt^n$$

where N_0 = the initial number of cells after come up time

N = the number of survival cells after an exposure time t.

b is the scale factor which is a characteristic of time and n is the shape factor. A small value of time (0.1) was used to approximate time=0.

Log-logistic model

A modified version of the original log-logistic model (Cole et al., 1993) with the following equation was used to fit the survivor curves obtained at 60°C.

$$\text{Log}\ \frac{N}{N_0} = \frac{\text{A}}{1 + \text{e}^{(-\sigma \log(\text{time}+1) - \tau}}$$

where A is the difference between lower and upper asymptotes, σ is the maximum rate of inactivation (maximum slope of inactivation curve) and τ is the log time to the maximum rate of inactivation.

The PROC NLIN procedure of SAS (SAS Institute, 2003) was used to the fit both the equations and to estimate the parameters for each treatment at a particular temperature. By using the estimated parameters in the equation of a specific model for a particular temperature-treatment combination, the time required for a specific log reduction was estimated by substituting the value for the targeted log reduction in place of the log reduction ratio (Log N/N_0). For the Weibull model, the time was estimated using the equation

$$t = \left(\frac{\text{Log } N/N_0}{b}\right)^{1/n}$$

where N_0 = the initial number of cells after come up time

N = the number of survival cells after an exposure time t.

b and n are parameters from Weibull model where b is the scale factor which is a characteristic of time and n is the shape factor (when n = 1, the curve is linear).

For the log-logistic model, the time was estimated by the following formula

$$t = (10^{\text{LN}(A/\ \text{Log } N/N_0 - 1)/(-\sigma) + \tau}) - 1$$

where A is the difference between lower and upper asymptotes, σ is the maximum rate of inactivation and τ is the log time to the maximum rate of inactivation.

The variance for the estimated time was calculated using the delta method (Billingsley, 1986). The values obtained for antimicrobial treatments at a particular temperature were compared using z statistics.

RESULTS AND DISCUSSION

Antimicrobial activity assay

Nisin activity as determined by critical dilution assay was 2.5×10^3 AU/mg. We used 2 mg of nisin/ml and thus the activity of nisin in the solution used was 5000 AU/ml. Lysozyme activity was 8 AU/mg and thus in the solution (10 mg/ml) used for the experiment, the activity was 80 AU/ml.

Temperature profile

The surface temperature profile of bologna samples was monitored throughout pasteurization for all three temperatures (Fig.4.1). Come up time, which is the time required for the bologna surface to reach the set water bath temperature, did not differ among three temperatures. The consistency in come-up times among samples was facilitated by the use of single slice bologna in all the thermal studies. The average come-up time was 60 s and surface temperature profiles did not fluctuate during the entire pasteurization process.

Initial inhibitory effect of antimicrobial treatments.

Nisin, and Nisin-lysozyme treatment resulted in a ~ 0.5- 0.6 log cfu/cm^2 reduction ($P < 0.05$) in cell counts almost immediately (at 0 h) compared to the control and lysozyme treatments (Table 4.1). The other three treatments did not differ ($P > 0.05$) in 0 time cell

counts and ranged from 7.38 (control) to 6.99 (nisin) log cfu/cm^2. There was no interaction effect ($P > 0.05$) between nisin and lysozyme indicating that there was an additive antibacterial effect for the nisin-lysozyme combination at 0 h. Previous studies reported a synergistic effect of nisin and lysozyme on spoilage as well as on pathogenic bacteria (Chung and Hancock, 2000; Gill and Holley, 2000; Nattress and Baker, 2003). Gill and Holley (2000) reported bactericidal action of a nisin and lysozyme (1:3) mixture and EDTA on the surface bologna and ham against *L. monocytogenes* when applied in a 7% gelatin coating. The short exposure time should be considered in the current study in discussing the small difference in reduction of *L. monocytogenes* due to the antimicrobial treatments. Extended exposure times will be examined in a subsequent study.

Bacterial lethality for the antimicrobial – heat treatment combination

Log reductions attained by the combined antimicrobial and heat treatment after a specific duration of heating at 3 pasteurization temperatures are given in Table 4.2.

The cut off point for duration of heating for each temperature was selected as the time interval at which there were detectable cells for all four treatments, not the complete duration of pasteurization used in this study (Antimicrobial treatments resulted in reduction of *L. monocytogenes* cells to below detection levels at 60 and 65°C by the completion of pasteurization for 720 and 60 s respectively). At 60°C, nisin and the nisin-lysozyme treatments resulted in a greater log reduction ($P < 0.05$) compared to control and lysozyme treatments after 9 min (540 s) of pasteurization (including come-up time). Nisin and nisin-lysozyme treatment resulted in a greater log reduction ($P < 0.05$) in *L. monocytogenes* counts compared to the control and lysozyme treatments when heated for 3 min (including come-up time) at 62.5°C. At 65°C, all antimicrobial treatments resulted

in a significant bacterial reduction ($P < 0.05$) compared to the control sample. The reductions obtained for control, lysozyme, nisin and nisin-lysozyme treatments were 4.02, 4.81, 5.54, and 5.83 log cfu/cm^2 respectively. Nisin and nisin-lysozyme treatments also resulted in higher reductions ($P < 0.05$) compared to the lysozyme treatment.

For all three pasteurization temperatures used, the nisin-lysozyme treatment resulted in lower ($P < 0.05$) *L. monocytognes* populations compared to the control and lysozyme treatments. At 65°C, the nisin treatment also reduced ($P < 0.05$) *L. monocytogenes* population on bologna compared to all treatments not containing nisin. Synergistic antibacterial activity of the combination of nisin and heat in various food matrices and laboratory media was reported by various research workers (Ueckert et al., 1998; Budu-Amako et al., 1999; Modi et al., 2000). Budu-Amako et al. (1999) investigated the combined effect of nisin and moderate heat to increase the killing of *L .monocytogenes* in cans of cold-pack lobster. Addition of nisin at a level of 25 mg/kg of can contents to the brine surrounding the lobster, in combination with a heat process giving internal can temperatures of 60°C for 5 min and 65°C for 2 min, resulted in 3-5 log reductions whereas heat or nisin alone resulted in decimal reductions of 1 to 3 logs. A synergy of nisin-lysozyme mixtures and heat in reducing *L. monocytogenes* populations was observed in the present study. The nisin-lysozyme treatment with heat resulted in an additional 1 log cfu/cm^2 reduction after 100 s of pasteurization at 65°C compared to the bactericidal effect obtained without heat treatment. Chung and Hancock (2000) attributed the increased efficacy of the mixture of nisin and lysozyme to increased membrane damage, cell lysis, or the inhibition of energy dependent processes that repair

nisin/lysozyme damage to the cell. The added inhibitory effect of this mixture with heat might be attributed to the additional changes in membrane permeability caused by heat. The enhanced inhibitory effect of in-package pasteurization combined with various antimicrobial agents was investigated in previous studies (Chen et al., 2004; Murphy et al. 2004; McCormick et al., 2005). Chen et al. (2004) combined the surface application of pediocin (ALTA 2341) with post-packaging thermal pasteurization in hot water at 71, 81, or 96°C for 30, 60, or 120 s to control *L. monocytogenes* on frankfurters. *L. monocytogenes* populations were reduced by all treatments, but 81°C or higher temperatures for at least 60 s in combination with pediocin were necessary to achieve at least 50% reduction of initial populations. In the present study, nisin and nisin-lysozyme treatments resulted in reduction of *L. monocytogenes* cells to below detectable levels on the bologna surface after pasteurizing for 60 s at 65°C. Food product type, packaging materials, and antimicrobial agents influence the thermal inactivation of the organism during in-package pasteurization and are likely the reason for differences in inactivation levels from similar applications.

There was an obvious effect of the three processing temperatures used in this study on the inactivation of *L. monocytogenes.* To obtain a reduction of 4-5 log cfu/cm^2 for various treatments used in the study, the time taken was about 3 fold longer at 60°C compared to 62.5°C, about 2 fold longer at 62.5°C compared to 65°C and more than 5 times longer at 60°C compared to 65°C.

Survival of *Listeria monocytogenes* on the bologna surface after pasteurization

Survivor curves for *L. monocytogenes* at the three temperatures tested were obtained by plotting log cfu/cm^2 versus the pasteurization time in seconds. The time scale started

when the bologna surface temperature reached the set water bath temperature (0 time). Total duration of pasteurization after reaching the come-up time at 65°C was 60 s with a dwell time of 10 s (Fig. 4.2). At 0 time, the *L. monocytogenes* population for antimicrobial treatments were lower than the control sample. This reduction was resulted from the bactericidal action of the antimicrobial treatments and also from the effect of heat during come-up. At 65°C, there were no viable *L. monocytogenes* cells recovered at 50 s for nisin and nisin-lysozyme treated samples and no cells were recovered at 60 s for lysozyme treated samples. At 62.5°C, duration of heating after reaching the come-up time was 120 s (Fig. 4.3). There was a reduction in bacterial population for nisin and nisin-lysozyme treatments at 0 time. However, beyond this point, all 4 treatments displayed a similar pattern. At 60°C, in order to have enough survival points in the curve, the duration of heating after come-up time was extended to 720 s (Fig. 4.4). Again, nisin and nisin-lysozyme treatments resulted in reduction of *L .monocytogenes* at 0 time. There were no detectable cells for any of the antimicrobial treatments at the end of pasteurization (720 s); however, the nisin-lysozyme treated bologna attained the no detectable *L. monocytogenes* threshold by 600 s.

Modeling of inactivation curves

Statistical analysis of the survivor data showed a non linearity and therefore a simple first-order linear model was not appropriate to model the bacterial death time relationship. Curves for some treatments showed a shoulder and also a small degree of tailing. The most common approach to describe microbial inactivation by thermal processing is to assume first-order kinetics. However, a first-order kinetics model is not compatible with curvature, a shoulder, or tailing of the survival curve (Virto et al., 2006).

The first-order approach to thermobacteriology assumes that each microorganism has the same probability of dying (van Boekel, 2002). In the present study, since antimicrobial agents were applied on the bologna surface before exposure to heat, the population of *L. monocytognes* at the beginning of heating could consist of cells with varying sensitivities to heat leading to a non-linear curve due to these differences in heat sensitivities.

The model that best fit the survivor curves obtained for 62.5 and 65°C was based on the Weibull distribution (Figs. 4.5 and 4.6). This is a very flexible model since it allows describing survivor curves showing a shoulder, a tail or even a linear behavior. Two parameters of this model, b and n are scale and shape parameters respectively. The estimates obtained for these parameters are given in Table 4.3. A $n<1$ corresponds to a concave upward survival curve, $n>1$ to a concave downward curve and n equal to 1 to a straight line. In the present study all of the treatments yielded a value of $n<1$ indicating an upward concavity of the curve. But values of the estimate of n in all cases were close to 1 (0.75-0.95) indicating that the upward concavity was minimal. Upward concavity can be interpreted as evidence that weak or sensitive members of the population are destroyed at a relatively fast rate leaving behind survivors of higher resistance (Peleg, 2000). But in this study, nisin and nisin-lysozyme treatments reduced the *L. monocytogenes* population to below detection levels indicating that there were only a minimum number of cells with higher heat resistance. This is also supported by the fact that there was no evidence of tailing which indicates the presence of heat resistance survivors at the end of the pasteurization. Massachalk et al. (2000) reported that a combination of nisin and lysozyme was helpful in reducing the tailing of high pressure survivor curves due to its ability to reduce the fraction of cells that survive the treatment compared to the use of

nisin and lysozyme separately. The parameter b did not show any systematic pattern among treatments nor between two pasteurization temperatures. According to Mafart et al. (2002) parameter b has no immediate physical significance. Rajan et al. (2006) used the Weibull model to fit the survivor curves while studying the inactivation of *Bacillus stearothermophilus* spores in egg patties by pressure assisted thermal processing, and observed that parameter b of the Weibull model increased for a treatment that yielded a greater log reduction. At 65°C, all of the antimicrobial treatments yielded b values more than 2-times that of the control treatment.

A modified log-logistic model was the best fit for the survivor curves at 60°C (Fig. 4.7). The parameter estimates obtained for this model are given in Table 4.3. The values of σ which is the maximum rate of inactivation (maximum slope of inactivation curve) and τ which is the log time to the maximum rate of inactivation did not show any obvious pattern among antimicrobial treatments. Stephens et al. (1994) reported a linear increase in τ with decrease in temperature. But in the present study, the model was fitted to curves obtained during heating at a constant temperature (60°C). Raso et al. (2000) used log-logistic model to predict inactivation of *Salmonella senftenberg* by pulsed electric fields and found a quadratic relationship between τ value and the electric field strength of treatment. While the Weibull model fits data that shoulders or tails but not data that displays both upper and lower asymptotes. The log-logistic model does fit asymptotic data which is what was found at 60°C.

Even though two models used in this study fit the bacterial heat inactivation very closely, the parameters of this model did not directly give a clear indication of inactivation kinetics. The influence of antimicrobial treatments on the parameters of the

models was not conclusive. A more useful measure of thermal processing to the industry would be the number of log reductions of the target organism achieved by the process and the time needed to achieve a targeted log reduction. This point was also raised in the Institute of Food Technologists' (IFT) second research summit to advance the understanding of microbial inactivation kinetics and models for non-log-linear survivor curves. It was suggested that "the performance of food preservation processes should be communicated in terms of the number of log cycles of reduction that the process is expected to deliver for the microorganism of concern rather than the D-value" (Heldman and Newsome, 2003). Therefore by using the estimated parameters in the equation of the specific model, and by substituting the required value for the decimal reduction ratio in the equation, the time required for specific log reduction was calculated. Calculated time of first decimal reduction (time required for initial decimal reduction) which is restricted to first decimal reduction of surviving cells from N_0 to $N_0/10$ is given in Table 4.4. In case of survivor curves, where a Weibull model was fitted, when the value of the parameter n is close to 1, the curve is close to a linear one and the estimate of time of first decimal reduction will be closer to an estimate of the D value used in first-order kinetics. When survivor curves are linear, the estimate of time of first decimal reduction allows comparison of microbial resistance because the inactivation rate is constant and independent of the pasteurization time (Virto et al., 2006). Since the survivor curves obtained in this study were not linear, the values of time of first decimal reduction are not suitable for comparing the effect of various antimicrobial treatments on the heat resistance of the organism.

A more useful measure would be the estimation of the time required for a targeted log reduction required to completely eliminate or reduce the *Listeria* population to a very low level. Again, since the curves were non-linear, extrapolating the targeted log reduction beyond the maximum reduction obtained in this experiment would not be ideal. Therefore considering the flexibility of both the models used in this study, the time required for a 4 log reduction in *L. monocytogenes* population was calculated (Table 5). The data obtained at 62.5 and 60°C were adequate to accurately determine 5-6 log reductions using the Weibull model parameters whereas at 60°C, the data could not be accurately extrapolated beyond a 4 log reduction due to the asymptotic nature of the logistic curve (lower asymptote). At 60°C, none of the antimicrobial treatments resulted in a significant difference in the time required to achieve a 4 log reduction compared to the control sample. At 62.5°C, nisin and nisin-lysozyme treatments resulted in a significant reduction ($P < 0.05$) in the 4 log reduction time compared to control and lysozyme treatments. The time required for 4 log reduction was 149.07, 138.15, 123.63, and 115.49 s for control, lysozyme, nisin, and nisin-lysozyme treatments, respectively. The nisin-lysozyme treatment required about 35 s less than the control sample to achieve 4 log reduction at 62.5°C. The time required for 4 log reduction in *L. monocytogenes* population at 65°C was 66.74, 65.47, 55.48, and 45.79 s for control, lysozyme, nisin, and nisin-lysozyme treatments, respectively. Nisin and nisin-lysozyme treatments resulted in a reduction ($P < 0.05$) in the time compared to control and lysozyme treatments. Nisin-lysozyme treatment required about 21 s less than the control sample to achieve the targeted reduction at 65 °C. At 62.5 and 65°C, the time required for a 5 log reduction

showed a similar trend as was seen for the 4 log reduction for the antimicrobial treatments (Data not shown).

The estimated values for the time required for 4 log reduction at 62.5 and 65°C indicated that pre-surface application of nisin and the nisin-lysozyme combination with in-package pasteurization was effective in reducing the time required for a targeted log reduction. With a higher concentration of the antimicrobial agents than the ones used in this study, this effect may be more pronounced. In the present study, the concentrations of the antimicrobial agents used were optimized (reduced) to obtain log reduction points in a range along with the control sample to allow meaningful comparisons. At 60 °C, the lowest of the temperature used in the study, the time estimates obtained for any of the antimicrobial treatments were not different ($P > 0.05$) from the control sample. This may be due to the fact that the synergy of these antimicrobials with heat was less pronounced at the lower temperature.

In previous studies where a first order kinetics model was fitted to obtain a decimal reduction time values (D-values), researchers have reported the ability of nisin and other antimicrobial agents in reducing the D value. A study by Wandling et al. (1999) found that the addition of 2000 IU of nisin per ml of skim milk lowered ($P < 0.05$) the D value of *Bacillus cereus* spores compared to a control sample. 2000 IU of nisin/ml resulted in a D value of 4.8 min whereas the D value for the control sample was 7.0 min. In the same study, for *Bacillus stearothermophilus* spores, 2000 IU of nisin /ml of skim milk lowered the D value from 16.0 (control) to 13.8 s. Knight et al. (1999) reported that nisin at a concentration 10µg/ml (40 IU/ml) significantly reduced the D value of *L. monocytogenes* in liquid whole egg at lower pasteurization temperatures (<58°C). From a practical point

of view, results form these two studies also suggest that nisin treatment resulted in significant reduction in the time required for a targeted log reduction.

CONCLUSIONS

Results from the present study indicate that pre-surface application of nisin and a combination of nisin and lysozyme (1:5) was effective in reducing the time required for a targeted log reduction in *L. monocytogenes* populations on the RTE bologna surface at 62.5 and 65°C. Another advantage of combining these two methods (heat + antimicrobial agents) is the possibility of reducing the concentration levels of the antimicrobial agents required to achieve adequate lethality compared to concentrations required to attain the same lethality when used without heat. Furthermore, the most aggressive USDA post-lethality option includes the use of an inhibitor for *L. monocytogenes* outgrowth during storage and application of nisin or nisin-lysozyme may also meet this guideline. Results from this study may be useful to the industry in developing a cost-effective in-package pasteurization process. Shorter duration of pasteurization will also help to preserve the desirable qualities of the food product. Future studies are needed to determine the antilisterial activity of this combined method on various RTE poultry products with different compositions, thicknesses and other characteristics.

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Table 4.1. The mean *Listeria monocytogenes* population (log cfu/cm^2) on inoculated bologna slices at 0 h for different treatments before subjecting to in-package pasteurization.

Treatment	Bacterial count (mean ± S.D)
Control[1]	7.38 ± 0.06^{a}
Lysozyme	7.31 ± 0.16^{ab}
Nisin	6.99 ± 0.15^{b}
Nisin - Lysozyme	6.71 ± 0.21^{bc}

[a,b,c]Means with different superscripts are significantly different ($P < 0.05$).

[1]Control = no antimicrobial added
Lysozyme = 0.1 ml of a 10 mg/ml (80 AU/ml) solution
Nisin = 0.1 ml of a 2mg/ml (5000 AU/ml) solution
Nisin-Lysozyme = 0.1 ml of a 2mg nisin + 10mg lysozyme/ml solution.

Each solution spread onto the 4 × 4 cm bologna surface of a 2.5 mm thick slice.

Table 4.2. The mean reductions* (log cfu/cm^2) in *Listeria monocytogenes* populations by combined antimicrobial and heat treatment after specific duration of heating (including 60 s come-up time) for 3 different pasteurization temperatures.

Temperature	Time	Treatment	Bacterial reductions (mean ± S.D)
60°C	9 min (540 s)	Control[1]	3.93 ± 0.61[a]
		Lysozyme	4.17 ± 0.38[a]
		Nisin	4.91 ± 0.25[b]
		Nisin-Lysozyme	5.36 ± 0.13[b]
62.5°C	3 min (180 s)	Control	3.83 ± 0.35[a]
		Lysozyme	4.10 ± 0.47[a]
		Nisin	4.99 ± 0.30[b]
		Nisin-Lysozyme	5.27 ± 0.29[b]
65°C	1.6 min (100 s)	Control	4.02 ± 0.31[a]
		Lysozyme	4.81 ± 0.11[b]
		Nisin	5.54 ± 0.18[c]
		Nisin-Lysozyme	5.83 ± 0.02[c]

* Log reductions attained by the combined antimicrobial and heat treatments after a specific duration of heating were calculated by subtracting the log count for each treatment after the duration from the initial log count obtained for the untreated control sample before heating.

[a,b,c]Means within each temperature with different superscripts are significantly different ($P < 0.05$).

[1]Control = no antimicrobial added
Lysozyme = 0.1 ml of a 10 mg/ml (80 AU/ml) solution
Nisin = 0.1 ml of a 2mg/ml (5000 AU/ml) solution
Nisin-Lysozyme = 0.1 ml of a 2mg nisin + 10mg lysozyme/ml solution.

Each solution spread onto the 4 × 4 cm bologna surface of a 2.5 mm thick slice.

Table 4.3. Parameters of Weibull and log-logistic models for antimicrobial treatments at different temperatures.

Temperature	Treatment	Weibull		Log-logistic	
		b	n	σ	τ
60°C	Control[1]			2.987	2.582
	Lysozyme	-		2.545	2.984
	Nisin			2.437	2.698
	Nisin-Lysozyme			6.827	2.433
62.5°C	Control	0.067	0.815		
	Lysozyme	0.038	0.942	-	
	Nisin	0.066	0.853		
	Nisin-Lysozyme	0.042	0.959		
65°C	Control	0.074	0.951		
	Lysozyme	0.172	0.752	-	
	Nisin	0.168	0.788		
	Nisin-Lysozyme	0.174	0.819		

[1]Control = no antimicrobial added
Lysozyme = 0.1 ml of a 10 mg/ml (80 AU/ml) solution
Nisin = 0.1 ml of a 2mg/ml (5000 AU/ml) solution
Nisin-Lysozyme = 0.1 ml of a 2mg nisin + 10mg lysozyme/ml solution.

Each solution spread onto the 4 × 4 cm bologna surface of a 2.5 mm thick slice.

Table 4.4. Time (seconds) for first 1 log cfu/cm^2 reduction in *Listeria monocytogenes* population on the surface of turkey bologna subjected to in-package pasteurization at different temperatures.

Temperature	Treatment	Time (Mean ± S.D)
60°C	Control[1]	97.13 ± 15.30[a]
	Lysozyme	101.89 ± 13.03[a]
	Nisin	80.26 ± 8.46[a]
	Nisin-Lysozyme	175.22 ± 9.22[b]
62.5°C	Control	27.23 ± 3.09[a]
	Lysozyme	31.73 ± 3.35[a]
	Nisin	23.98 ± 2.25[a]
	Nisin-Lysozyme	27.24 ± 2.53[a]
65°C	Control	15.51 ± 1.63[a]
	Lysozyme	10.36 ± 1.62[b]
	Nisin	9.57 ± 0.85[b]
	Nisin-Lysozyme	8.43 ± 1.64[b]

[a,b]Means with different superscripts within each temperature are significantly different ($P < 0.05$).

[1]Control = no antimicrobial added
Lysozyme = 0.1 ml of a 10 mg/ml (80 AU/ml) solution
Nisin = 0.1 ml of a 2mg/ml (5000 AU/ml) solution
Nisin-Lysozyme = 0.1 ml of a 2mg nisin + 10mg lysozyme/ml solution.

Each solution spread onto the 4 × 4 cm bologna surface of a 2.5 mm thick slice.

Table 4.5. Time (seconds) required for 4 log cfu/cm^2 reduction in *Listeria monocytogenes* population on the surface of turkey bologna subjected to in-package pasteurization.

Temperature	Treatment	Time (Mean ± S.D)
60°C	Control[1]	497.29 ± 22.45[a]
	Lysozyme	462.22 ± 12.34[a]
	Nisin	519.10 ± 12.43[a]
	Nisin-Lysozyme	515.18 ± 47.68[a]
62.5°C	Control	149.10 ± 9.68[a]
	Lysozyme	138.15 ± 10.10[a]
	Nisin	123.63 ± 4.86[b]
	Nisin-Lysozyme	115.49 ± 6.75[b]
65°C	Control	66.74 ± 3.30[a]
	Lysozyme	65.47 ± 4.75[a]
	Nisin	55.48 ± 2.69[b]
	Nisin-Lysozyme	45.79 ± 3.34[b]

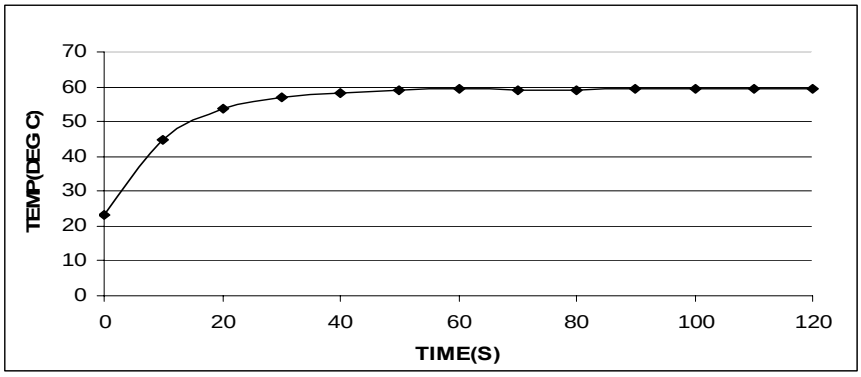
[a,b]Means with different superscripts within each temperature are significantly different ($P < 0.05$).

[1]Control = no antimicrobial added
Lysozyme = 0.1 ml of a 10 mg/ml (80 AU/ml) solution
Nisin = 0.1 ml of a 2mg/ml (5000 AU/ml) solution
Nisin-Lysozyme = 0.1 ml of a 2mg nisin + 10mg lysozyme/ml solution.

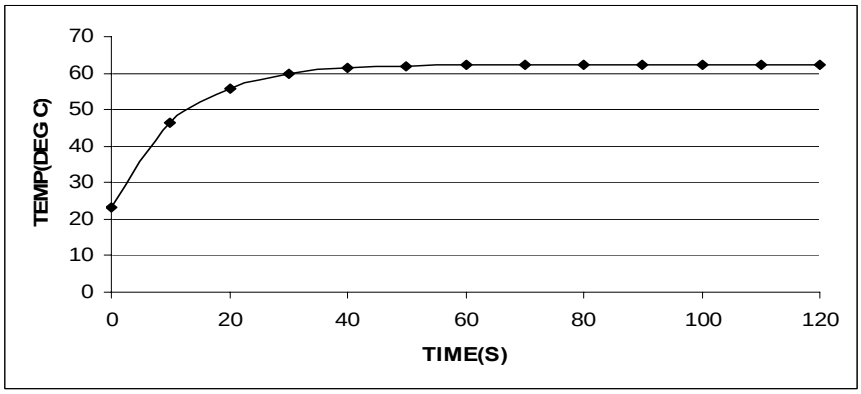
Each solution spread onto the 4 × 4 cm bologna surface of a 2.5 mm thick slice.

Fig. 4.1. Surface temperature profile of bologna during in-package pasteurization.

60°C



62.5°C



65°C

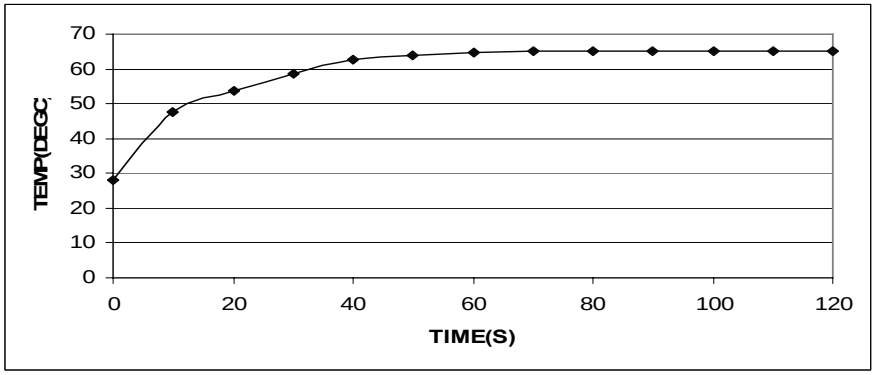


Fig. 4.2. Survivor curves for *Listeria monocytogenes* on bologna surface at 65°C for various antimicrobial treatments. Each data point represents the mean of three experimental replications. Standard deviation ranged between 0.01-0.6 log cfu/cm^2.

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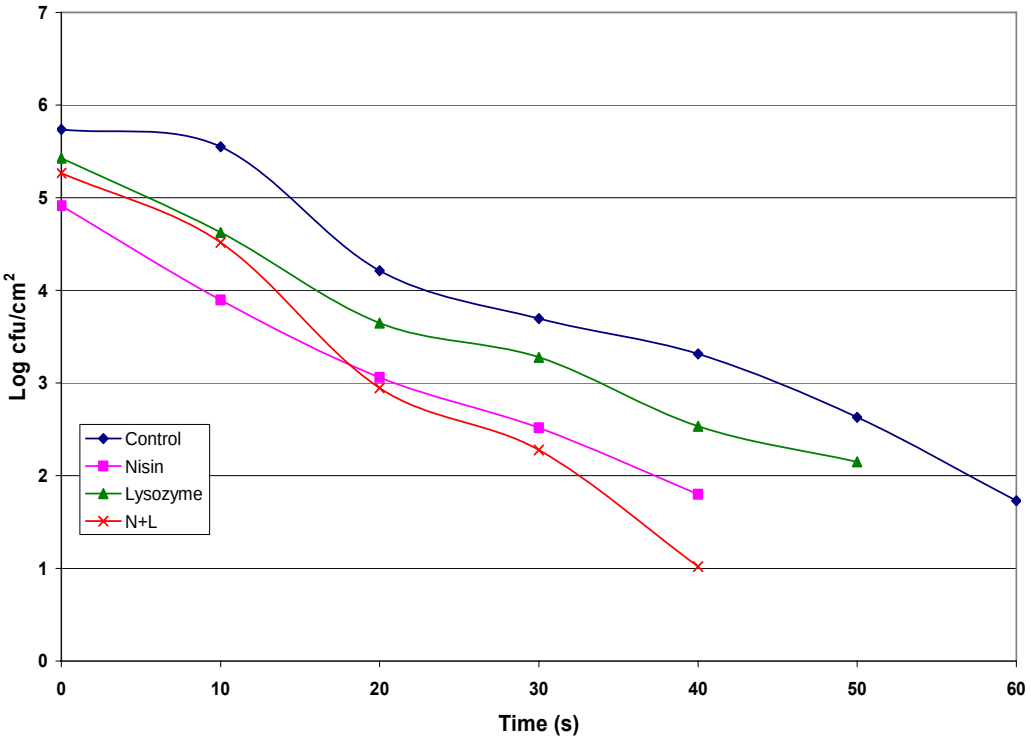


Fig. 4.3. Survivor curves for *Listeria monocytogenes* on bologna surface at 62.5°C for various antimicrobial treatments. Each data point represents the mean of three experimental replications. Standard deviation ranged between 0.05-0.7 log cfu/cm^2.

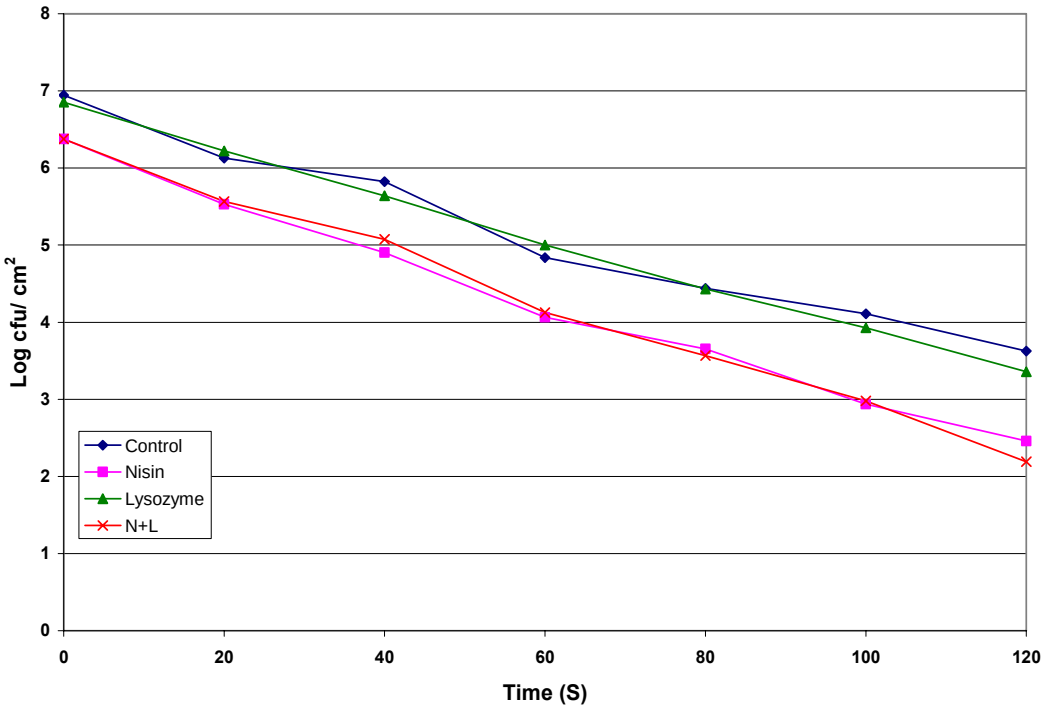


Fig. 4.4. Survivor curves for *Listeria monocytogenes* on bologna surface at 60°C for various antimicrobial treatments. Each data point represents the mean of three experimental replications. Standard deviation ranged between 0.03-0.8 log cfu/cm^2.

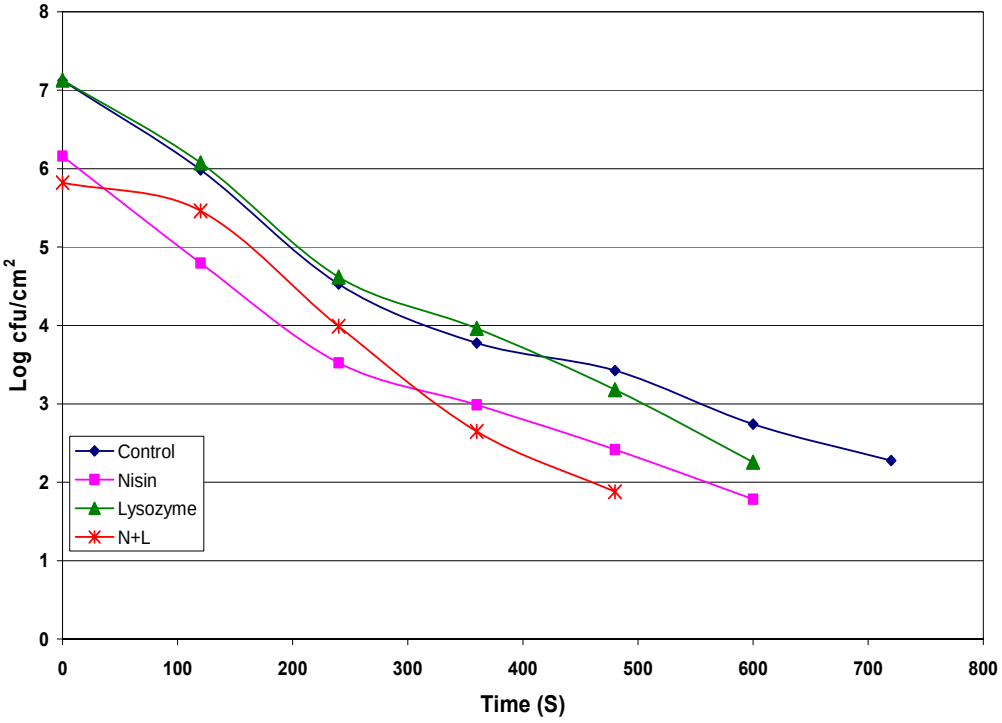
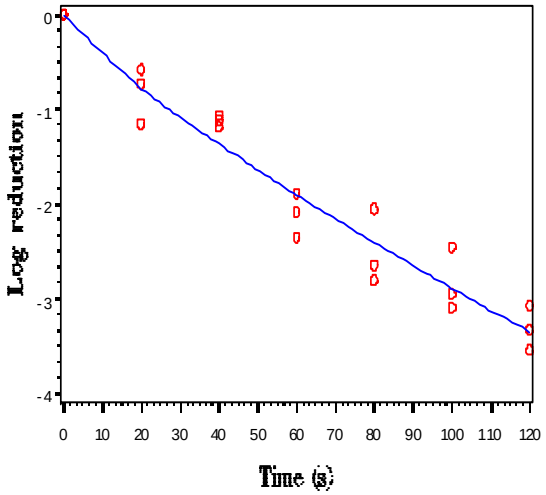


Fig. 4.5. Weibull model fitted to the survivor curves of various treatments at 62.5°C.

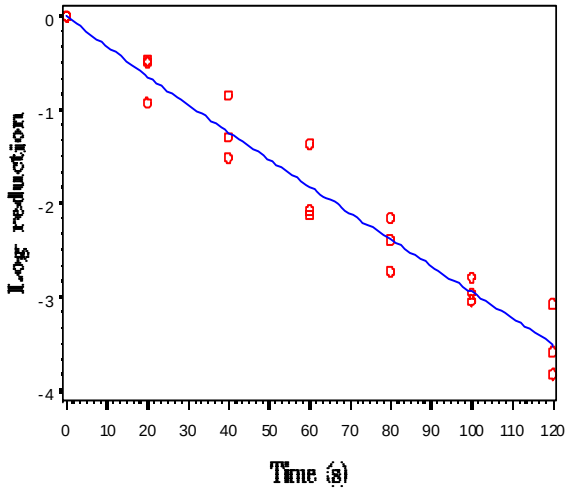
CONTROL

b = 0.067 n = 0.815



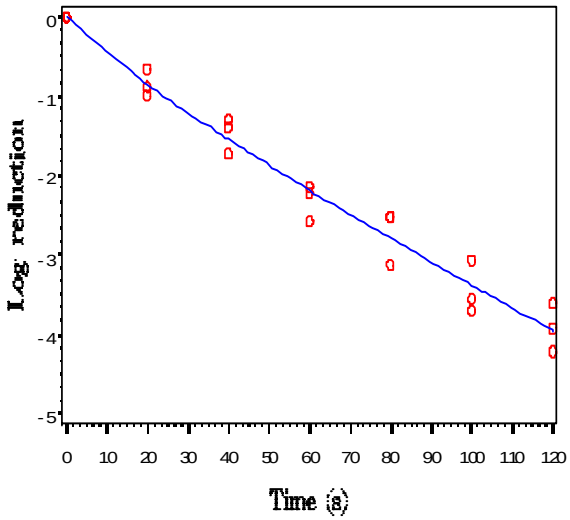
LYSOZYME

b = 0.038 n = 0.942



NISIN

b = 0.066 n = 0.853



NISIN-LYSOZYME

b = 0.042 n = 0.959

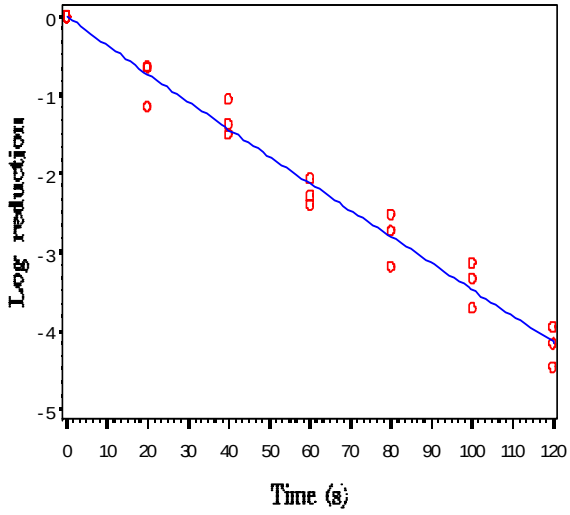
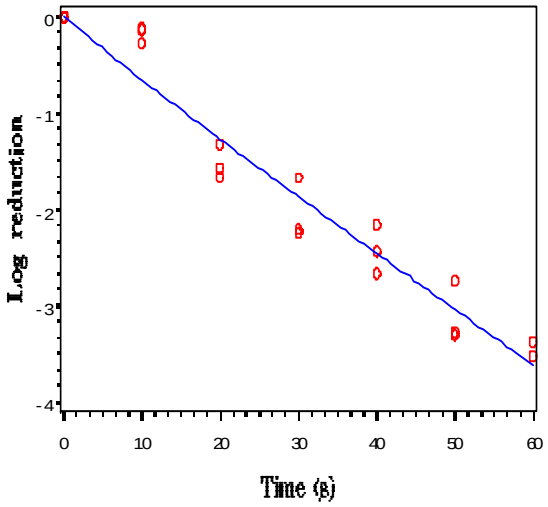


Fig. 4.6. Weibull model fitted to survivor curves of various treatments at 65°C.

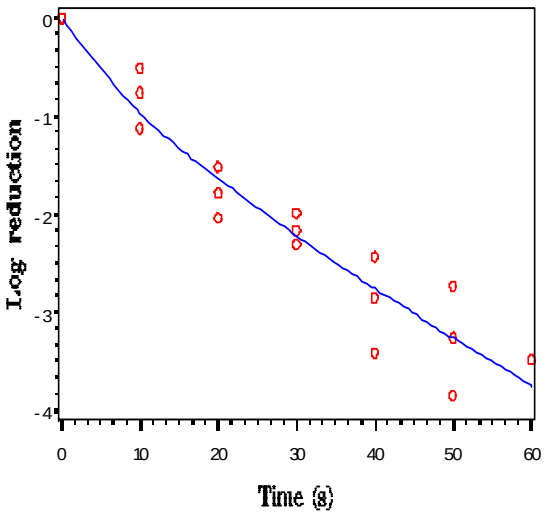
CONTROL

b = 0.074 n = 0.951



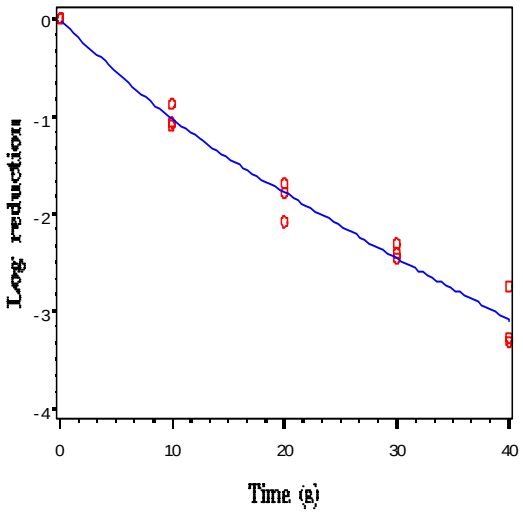
LYSOZYME

b = 0.172 n = 0.752



NISIN

b = 0.168 n = 0.788



NISIN-LYSOZYME

b = 0.174 n = 0.819

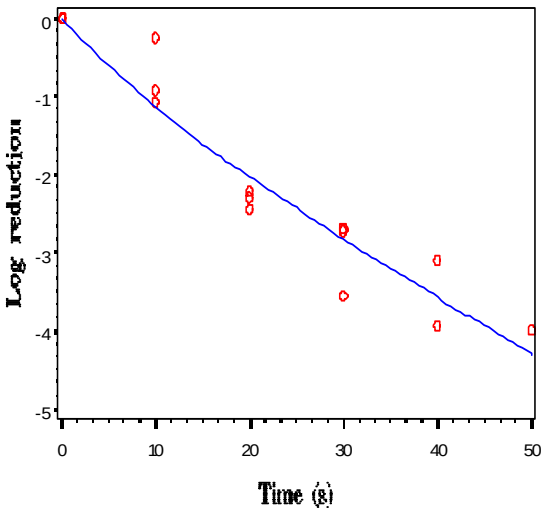
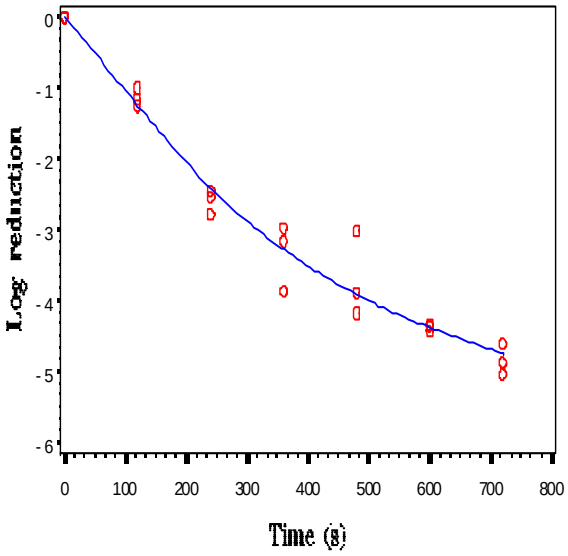


Fig. 4.7. Log-logistic model fitted to survivor curves of various treatments at 60°C.

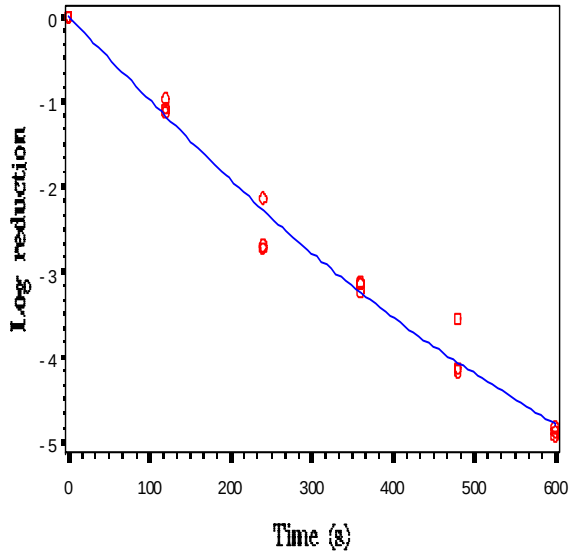
CONTROL

$\sigma = 2.987$ $\tau = 2.582$



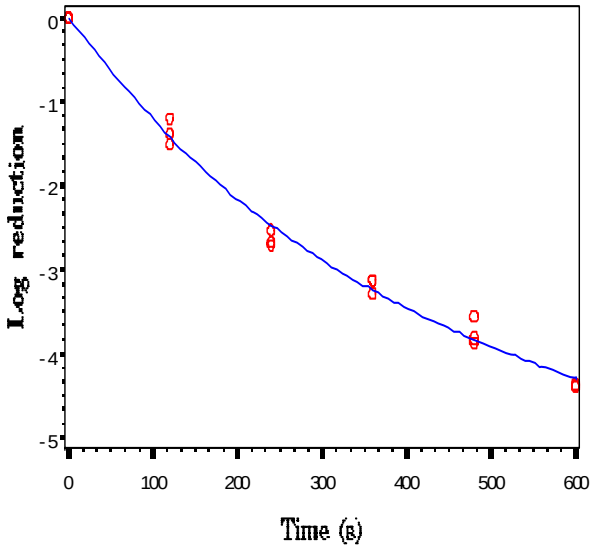
LYSOZYME

$\sigma = 2.545$ $\tau = 2.984$



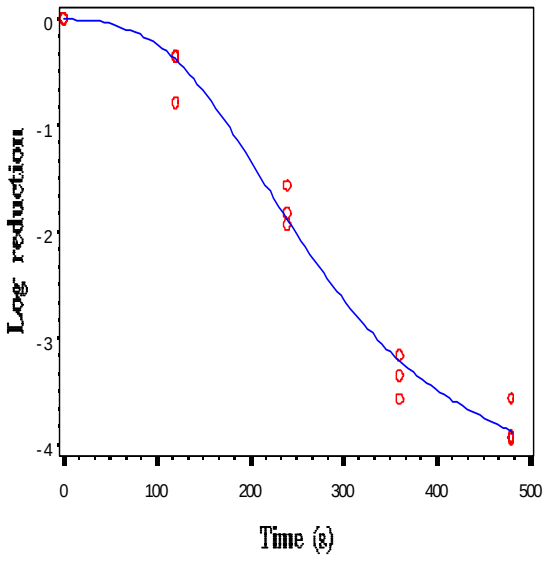
NISIN

$\sigma = 2.437$ $\tau = 2.698$



NISIN-LYSOZYME

$\sigma = 6.827$ $\tau = 2.433$



CHAPTER 5
EFEECT OF COMBINING NISIN AND/OR LYSOZYME WITH IN-PACKAGE PASTEURIZATION FOR CONTROL OF *LISTERIA MONOCYTOGENES* IN READY-TO-EAT TURKEY BOLOGNA DURING REFRIGERATED STORAGE

ABSTRACT

Post-processing contamination of ready-to-eat (RTE) meat and poultry products by *L. monocytogenes* is a major food safety issue. Because of the ability of *L. monocytogenes* to grow and multiply at refrigeration temperatures, even a low level of initial contamination can result in a substantial number on the food product at the time of consumption. This study investigated the efficacy of in-package pasteurization combined with pre-surface application of nisin and/or lysozyme to reduce and prevent the subsequent recovery and growth of *L. monocytogenes* during refrigerated storage on the surface of low-fat turkey bologna. Sterile bologna samples were treated with solutions of nisin (2mg/ml = 5000 AU/ml), lysozyme (10mg/ml = 80 AU/ml) and a mixture of nisin and lysozyme (2mg nisin+ 10mg lysozyme/ml) before in-package pasteurization at 65°C for 32 s. In-package pasteurization resulted in an immediate 3.5-4.2 log cfu/cm^2 reduction in *L. monocytogenes* population for all treatments. All pasteurized treatments also resulted in a significant reduction of *L. monocytogenes* by 12 weeks compared to un-pasteurized bologna. In-package pasteurization in combination with nisin or nisin-lysozyme treatments was effective in reducing the population below detectable levels by 2-3 weeks of storage. Results from this study could have a significant impact for the

industry since a reduction in bacterial population was achieved by a relatively short pasteurization time and antimicrobials reduced populations further during refrigerated storage.

INTRODUCTION

Listeria monocytogenes is a pathogenic bacterium causing listeriosis and is considered as an important worldwide public health problem. Incidence of listeriosis in developed countries ranges from 4 to 8 cases per 1,000,000 individuals (FAO/WHO, 2000). The mortality rate for listeriosis averages 30% compared to 0.38% and 0.1% for Salmonellosis and Campylobacteriosis, respectively (Wing and Gregory, 2002). About 99% of listeriosis cases reported in the United States had a foodborne source (Mead et al., 1999). Control of *L. monocytogenes* growth in food is problematic since the organism can grow at temperatures ranging from 1 to 45°C, tolerate high salt concentrations, and initiate growth at a relatively low pH (Vignolo et al., 2000). Foods considered as high-risk sources of listeriosis include foods which are ready-to-eat, require refrigeration, and are stored for extended time periods (ILSI research foundation, 2005). These food types have potential for *L. monocytogenes* contamination and can support its growth to dangerous levels. Recent listeriosis outbreaks have been epidemiologically linked to deli meats and RTE meat and poultry products. A majority of food product recalls associated with *L. monocytogenes* contamination involve RTE meat and poultry products (FSIS, 2005). Because of the public health significance of this organism and high susceptibility of RTE meat products for contamination and growth, the United States Department of Agriculture (USDA) implemented a “zero-tolerance” policy for this pathogen in RTE meat products (USDA, 1989).

L. monocytogenes is often present in meat processing environments. Routine sanitation steps are sometimes not effective in eliminating this organism from the processing environment because of biofilm formation on various surfaces (Houben and Eckenhausen, 2006). This organism can contaminate RTE meat and poultry products during post-lethality steps such as slicing, peeling, and packaging (Murphy et al., 2005). Post-processing contamination of RTE meat products with *L. monocytogenes* is of even greater concern because often these products are consumed without additional heating. In an effort to control post-processing *L. monocytogenes* contamination of RTE meat and poultry products, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) established an interim final rule in 2003 (USDA FSIS, 2003). The establishments that produce RTE meat and poultry products are required to comply with one of three alternatives proposed by this rule. Alternatives 1 and 2 require the use of a post lethality treatment to reduce or eliminate *L. monocytogenes* and/or antimicrobial agents to limit or suppress the growth of the organism throughout the storage, whereas alternative 3 relies on sanitation measures and testing to control the organism in the processing environment.

There have been a number of intervention methods tested to reduce *L. monocytogenes* population on RTE meat surfaces. These methods include surface pasteurization, high pressure processing, pulsed electric field, irradiation, and application of various antimicrobial agents. In-package pasteurization of RTE meat products using steam or hot water was useful in reducing the number of *L. monocytogenes* cells on the surface of RTE meat products (Roering et al., 1998; Muriana et al., 2002; McCormick et al., 2004). Muriana et al. (2002) reported a 2 to 4 log reduction of *L. monocytogenes* populations in

RTE deli-style vacuum-packaged whole or formed turkey, ham, and roast beef following post-package pasteurization at 90.6 to 96.1°C for10 min. McCormick et al. (2003) determined the D values of *L. monocytogenes* and *Salmonella* Typhimurium at various surface pasteurization temperatures for low fat turkey bologna slices. These researchers found that complete inactivation of *S.* Typhimurium and *L. monocytogenes* cells can be achieved using an in-package pasteurization process. A study to evaluate small-scale hot-water post packaging pasteurization as a post lethality treatment for *L. monocytogenes* on RTE beef snack sticks and natural-casing wieners by Ingham et al. (2005) found a ≥ 2 log reductions in bacterial populations when heated at 100°C for 1 min for individually packaged beef snack sticks and 4 min for packages of four and seven sticks.

Combining in-package pasteurization with antimicrobial treatments is a novel approach in food preservation (Samelis et al., 2002; Chen et al., 2004; McCormick et al., 2005). The combined process may have a greater impact on bacterial populations compared to interventions using a single treatment and help to reduce the needed intensity of the heat treatment. McCormick et al. (2005) tested the inhibitory effect of in-package pasteurization combined with a nisin-impregnated wheat gluten film over 8 weeks storage against *L. monocytogenes* and *S.* Typhimurium populations on refrigerated bologna. These researchers found that combining both treatments significantly reduced the *L. monocytogenes* population and prevented outgrowth over 8 weeks storage compared to the individual treatments.

Nisin is the most commonly used natural antimicrobial compound and was approved for use in food in 1969 and was awarded generally recognized as safe (GRAS) status in the United States in 1988 (FDA, 1988). Nisin is effective in a number of food systems,

inhibiting the growth of a wide range of Gram-positive bacteria, including many important foodborne pathogens such as *L. monocytogenes* (Tagg et al., 1976). Nisin has shown synergistic antibacterial activity with several compounds and processes.

Lysozyme is an enzyme that lyses the cell wall of certain Gram-positive bacteria. Hen egg white lysozyme is one of the few natural antimicrobials derived from an animal source that are applied as a food preservative. Synergistic antibacterial activity between nisin and lysozyme has been reported by various researchers (Chung and Hancock, 2000; Gill and Holley, 2000; Nattress and Baker, 2003).

L. monocytogenes is a psychrotrophic organism capable of survival and growth at refrigeration temperatures. Application of additional hurdles to control the growth of this organism would provide an increase margin of safety during refrigerated storage. Since refrigeration is a common method for storing RTE food products, understanding the survival and growth of *L. monocytogenes* at refrigeration temperatures in meat samples subjected to a combination of in-package pasteurization and antimicrobial treatments is important. Therefore the objective of this study was to evaluate the efficacy of surface application of nisin and/or lysozyme in combination with in-package pasteurization of RTE low fat turkey bologna against *L. monocytogenes* on the bologna surface during refrigerated storage (4°C) for 3 months.

MATERIALS AND METHODS

Food product preparation

Ready-to-eat low fat turkey bologna averaging 14.3% fat, 10.7% protein, and 71.4% moisture was used for the experiment. Bologna samples were batch irradiated for 521 min using a cobalt 60 source with a total dose of 2.4 Mrad at 4,607 R/minute at Auburn

University. Irradiation was carried out to eliminate background flora before inoculation studies. Bologna samples were kept frozen at -70°C and thawed overnight at 4°C prior to experimentation. For inoculation and thermal inactivation studies, bologna slices of approximately 2.5 mm thickness were cut into 4cm × 4cm pieces using a sterile cutting template and each piece was used as an experimental unit.

Culture preparation

Listeria monocytogenes ATCC 15313 was preserved by freezing the cultures at -70°C in vials containing brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 20% (v/v) glycerol (Sigma, St. Louis, MO). To propagate the culture, a frozen vial was thawed at room temperature, and 0.1 ml of the thawed culture was transferred to 9.9 ml of BHI broth in screw-cap tubes and incubated aerobically for 16-18 h at 37°C with agitation at 200 revolutions per min (Thermolyne Maxi-Mix III type 65800, Barnstead/Thermolyne, Dubuque, IA). The inoculum was prepared from a second transfer of this culture (0.1ml) to another 9.9 ml tube of BHI broth, and incubated aerobically for 16-18 h at 37°C with agitation. After incubating for 16 h, a washed cell suspension of the organism was prepared by harvesting the cells by centrifugation at 3000 × g (IEC HN-SII centrifuge, International Equipment Co., Inc., Needham Heights, MA), washing with 10 ml of 0.1% sterile peptone water (Bacto peptone, Difco Laboratories, Detroit, MI), and resuspending in 0.1% sterile peptone water to obtain a population of approximately 8- 9 $\log_{10}$ cfu/ml. Initial cell populations were verified by enumerating the cells after pour-plating in BHI agar and incubating at 37°C for 48 h.

Antimicrobial preparation

Nisin

Nisaplin, a commercial nisin product (2.5% nisin) was provided by Danisco (Danisco USA Inc, New Century, KS). Solutions of required concentrations of nisin were prepared on the day of the experiment by dissolving appropriate amount of nisin in sterile distilled water.

Lysozyme

Egg white lysozyme was provided by Q.P. Corporation (Q.P. Corporation, Japan). Solutions of lysozyme were prepared on the day of the experiment by dissolving appropriate amount in sterile distilled water.

Antimicrobial activity assay

Activities of nisin and lysozyme were determined by critical dilution assay (Pucci et al., 1988). Serial two-fold dilutions of the antimicrobial agents were tested against *L. monocytogenes* ATCC 15313. Ten µl of each dilution was spotted on the surface of the BHI agar medium seeded uniformly with a suspension of *L. monocytogenes*. After incubation (48h at 37°C), the plates were checked for zones of inhibition. Titer of nisin and lysozyme in Arbitrary Units per ml (AU/ml) was expressed as the reciprocal of the highest dilution showing a definite inhibition zone. Activity of the antimicrobial compounds was expressed in AU/mg based on the weight of the antimicrobial compounds used in serial dilution and converted into AU/ml based on the weight (mg) of the antimicrobial compound used in the application solution.

Antimicrobial treatment and inoculation

Sterile, frozen bologna samples were thawed overnight at refrigeration temperatures (4±1°C). On the day of the experiment, bologna samples were removed from refrigerator and aseptically transferred to a sterile surface under a Germfree ®Bioflow chamber (Ormond Beach, FL). Each bologna slice was cut into 4cm × 4cm pieces using a sterile cutting template as previously described. Meat samples were uniformly surface-treated with 0.1 ml of the antimicrobial solutions. The activity units applied for each treatment were control: 0, nisin: 500 AU (5000 AU/ml=2 mg/ml), lysozyme: 8 AU (80 AU/ml=10 mg/ml), and nisin-lysozyme: 508 AU (5000 AU nisin + 80 AU lysozyme/ml). After the antimicrobial treatment, all bologna surfaces were inoculated uniformly with a 0.1ml of a 9 $\log_{10}$ cfu/ml suspension of *L. monocytogenes* resulting in a population of 8 $\log_{10}$ cfu/slice. All samples were aseptically transferred to a post-pasteurization bag (Model CNP-310, Cryovac, Duncan, SC). Insulated thermocouples (K type Teflon, Omega Engineering, Inc, Stamford, CT) were attached to the surface of two un-inoculated bologna samples to measure the temperature during thermal treatment. All bags were then vacuum packaged (Koch Model, UV 250, Koch Supplies Inc, Kansas City, MO) (vacuum: 98, seal: 2, and gas : no.).

A set of all treatments were stored in a refrigerator at 4°C without subjecting to heat treatment for 12 weeks and another set was subjected to heat treatment before storage.

Thermal treatment/microbiological analysis

Bologna samples were exposed to 65°C for 32 s (After 60 s of come up time). This thermal treatment was targeted to achieve a 3 log reduction based on the results obtained in the previous part of this study (This target was set in order to have sufficient number of

surviving cells after the heat treatment to evaluate surviving cells during refrigerated storage). Vacuum-packaged bologna samples were simultaneously submerged into a water bath (Precision, model 186, Precision Scientific Incorporated, Chicago, USA) set to 65°C. The surface temperature and time data were monitored using a channel datalogger (CALPlex 32, TechniCAL, New Orleans, LA) and thermal processing software (CALSoft, Version 1.32, TechniCAL, New Orleans, LA) during the entire process. Once the bologna surface reached the treatment temperature, samples were held for 32 s, removed from the water bath, and rapidly cooled by immersing in ice-water slurry for 10 s to minimize further cell death, then stored in a refrigerator at 4°C for 12 weeks. Both pasteurized and non-pasteurized samples were tested on at day 0 and weeks 1, 2, 3, 4, 6, 8, 10, and 12 for *L. monocytogenes* populations. Samples were aseptically removed from the pasteurization bag and homogenized with 99 ml (20 ml for pasteurized samples since those samples had lower populations) of sterile 0.1% peptone water by placing the samples into a stomacher (Seward Stomacher 400 Circulator, Seward, Inc, UK) for 1 min at 230 rpm. Homogenates were then serially diluted and appropriate serial dilutions were pour plated in duplicate using BHI agar. Plates were incubated at 37°C for 48h before enumerating colonies. Colony forming units (cfu) counts were converted to $\log_{10}$ cfu/cm^2 of the sample before data analysis.

Statistical Analysis

The experiment was replicated 3 times on different days. Average *L. monocytogenes* populations during refrigerated storage for various treatments within pasteurized and non-pasteurized groups were compared using linear contrasts of treatment means. Where significant difference were detected ($P < 0.05$), treatment means were compared using the

LSD comparison procedure. The data were analyzed using the general linear model procedure of SAS (SAS Institute, 2003).

RESULTS AND DISCUSSION

Antimicrobial activity assay

Nisin activity as determined by critical dilution assay was 2.5×10^3 AU/mg. We used 2mg of nisin/ml and thus the activity of nisin in the solution used was 5000 AU/ml. Lysozyme activity was 8 AU/mg and thus in the solution (10mg/ml) used for the experiment, the activity was 80 AU/ml.

Initial inhibitory effect

Treatment of bologna surfaces with antimicrobial agents alone did not reduce ($P > 0.05$) the *L. monocytogenes* population at 0 day (Fig. 5.1). This may be due to the fact that the concentrations of antimicrobials used in the present study were not high enough to have an immediate bactericidal effect. We chose the antimicrobial concentrations in an attempt to elucidate any advantage of combining antimicrobial application with in-package pasteurization (with minimum intensities of both treatments) and also to have sufficient surviving cells after heat treatment to study their behavior during refrigerated storage. With this in mind, the lack of an initial reduction is not surprising. This also made any synergism between heat and antimicrobials more obvious.

In-package pasteurization at 65°C for 32 s reduced ($P < 0.05$) the *L. monocytogenes* population at 0 day for all four treatments used in this study (Fig. 5.1). For the control sample, the process reduced bacterial population from 7.6 to 4.1 log cfu/cm^2 and for lysozyme treatment from 7.4 to 3.5 log cfu/cm^2. In-package pasteurization of nisin and nisin-lysozyme treated samples resulted in a reduction from 7.0 to 3.2 and 6.9 to 2.7 log

cfu/cm^2, respectively. For pasteurized samples, at 0 day, the *L. monocytogenes* populations for nisin and nisin-lysozyme treated samples were lower ($P < 0.05$) than the control treatment. Nisin-lysozyme treatment had lower *L. monocytognes* populations ($P < 0.05$) compared to the lysozyme treatment. In-package pasteurization is an effective method to reduce *L. monocytogenes* populations on meat surfaces. Roering et al.(1998) found that hot water pasteurization of vacuum-sealed packages of summer sausages reduced a three-strain mixture of *L. monocytogenes* population by about 3 log cfu/g within 30, 60, and 90 s at 99, 88, and 77°C, respectively. Results from a study to evaluate the efficiency of hot water post-package pasteurization of RTE deli meats for reduction of *L. monocytogenes* by Muriana et al. (2002) indicate that minimal heating regimens of 2 min at 90.6 to 96.1°C can readily provide 2 log reductions in most RTE deli meats. The relatively long heating time reported by these research workers compared to the present study is probably due to differences in sample thickness. Mangalassary et al. (2004) found that surface heating rate during in-package pasteurization of turkey bologna was higher for lower product thickness levels. McCormick et al. (2005) found that in-package pasteurization of low fat turkey bologna at 65°C for 81 s resulted in a 4.8 log cfu/g reduction of *L. monocytognes* population. In the present study, in-package pasteurization at 65°C for 32 s resulted in a 3.5 log cfu/cm^2 reduction in *L. monocytogenes* population on the bologna surface for the control treatment. In the above mentioned study by McCormick et al. (2005) as well as in the present study, the use of a single slice of bologna with approximate thickness of 2.5 mm might have contributed to a higher surface heating rate and thereby a higher reduction of *L. monocytogenes* populations even

by a less intensive treatment at 65°C and for the differences in inactivation levels from similar applications described above.

Combining in-package pasteurization of meat products with antimicrobial agents has been tested for enhanced bactericidal activity by various research workers (Chen et al., 2004; Murphy et al., 2004; McCormick et al., 2005). In the present study, nisin-lysozyme treatment resulted in an additional reduction of 1.4 log cfu/cm^2 in *L. monocytogenes* population (2.7 log cfu/cm^2) immediately after in-package pasteurization compared to the control sample (Fig. 5.1). There was an additive inhibitory effect of nisin and lysozyme. In-package pasteurized bologna combined with no antimicrobial, lysozyme or nisin had 4.1, 3.4 and 3.2 log cfu/cm^2 *L. monocytogenes* populations, respectively. McCormick et al. (2005) reported an additional 1.5 log cfu/g reduction of *L. monocytogenes* population for bologna samples treated with a nisin containing film compared to a control sample when subjected to in-package pasteurization at 65°C for 81 s. Chen et al. (2004) combined the surface application of pediocin (ALTA 2341) with post-packaging thermal pasteurization in hot water at 71, 81, or 96°C for 30, 60, or 120 s to control *L. monocytogenes* on frankfurters. These researchers found that sample immersed in hot water at 96°C for at least 60 s or at 81°C for 120 s in combination with pediocin reduced the *L. monocytogenes* population to below detectable levels.

Survival of *Listeria monocytogenes* during refrigerated storage

For non-pasteurized bologna samples, the *L. monocytogenes* population remained relatively constant for each treatment during 12 weeks refrigerated storage (Fig.5.1) fluctuating between 7 to 8 log cfu/cm^2. There was no difference ($P < 0.05$) in the average *L. monocytogenes* populations for various treatments during 12 weeks of storage.

Absence of significant growth on non-pasteurized bologna surfaces during refrigerated storage could be attributed to vacuum packaging and the relatively high inoculation level since at 8 log cfu/cm^2, bacterial growth may be limited by the environment (nutrients, metabolites). Sheridan et al. (1995) reported that *Listeria* spp. did not grow in vacuum-packed lamb at 5°C. Absence of *L. monocytogenes* growth on vacuum packaged beef stored at 2, 4, 5 or 7°C were also reported by others (Kaya and Schmidt, 1991; Gibbs et al., 1993). In the present study, the concentration levels of antimicrobial treatments used were not high enough to reduce a relatively high initial concentration (7.58 log cfu/cm^2) of *L. monocytogenes* during storage. In a previous study by Schillinger et al. (2001), when nisin was used at a concentration of 1000 IU/ml, regrowth was prevented in tofu inoculated with a low number of *L. monocytogenes* (60 cfu/g) but not at a 10- fold higher inoculum. But nisin was effective in reducing the *L .monocytogenes* population level at a concentration of 5000 IU/ml in vacuum-packaged bologna and ham when an initial inoculum level of 3 to 4 log cfu/cm^2 was used (Geornaras et al., 2005).

Significant differences ($P < 0.05$) in *L. monocytogenes* populations were found among treatments at week 1 for pasteurized bologna samples. Nisin and nisin-lysozyme treatments had lower ($P < 0.05$) bacterial populations compared to control and lysozyme treatments. At week 2, no differences ($P > 0.05$) were found in *L. monocytogenes* populations among 4 treatments. In-package pasteurization of control samples resulted in a reduction ($P < 0.05$) of 2.4 log cfu/cm^2 in *L. monocytogenes* population by 12 weeks of storage (Fig. 5.1). Bacterial population reduced almost linearly for first 4 weeks (from 4.1 to 1.5 log cfu/cm^2), after which there was an increase ($P < 0.05$) at 6 weeks to 2.9 log cfu/cm^2. From 6 to 12 weeks, the population reduced ($P < 0.05$) to 2 log cfu/cm^2. Even

though in-package pasteurization was effective in reducing the *L. monocytogenes* population during refrigerated storage, it did not completely eliminate its presence with about 2 log cfu/cm^2 of cells remaining on the bologna surface after 12 weeks. This is probably due to the fact that there was no continuous inhibitory effect on the organism during storage as there was no additional inhibitory mechanism employed to suppress the growth. In-package pasteurization of lysozyme treated bologna samples resulted in a 1.9 log cfu/cm^2 reduction (from 3.5 to 1.6 log cfu/cm^2) of *L. monocytogenes* by 8 weeks of refrigerated storage, after which no cells were detected through 12 weeks (Fig. 5.1). There was a 1.7 log cfu/cm^2 reduction ($P < 0.05$) in *L. monocytogenes* populations (from 3.2 to 1.5 log cfu/cm^2) for nisin treated, pasteurized bologna during storage at 4°C from day 0 to week 3 (Fig. 5.1). After week 3, the *L. monocytogenes* population further declined to undetectable levels for the remainder of storage. Pasteurized nisin-lysozyme treatment reduced *L. monocytogenes* populations from 2.7 log cfu/cm^2 to 1.5 log cfu/cm^2 over 2 weeks of refrigerated storage (Fig. 5.1) and after week 2, further declined to undetectable levels through 12 weeks. These results support the premise of the USDA RTE meat *L. monocytogenes* intervention ruling that the use of inhibitor is effective in reducing bacterial population during storage. The combination of in-package pasteurization with antimicrobial treatments reduced bacterial populations below detectable levels by 9 weeks. McCormick et al. (2005) studied the inhibitory effects of in-package pasteurization at 65°C combined with a nisin (7% w/w) containing wheat gluten film over 8 weeks of storage against *L. monocytogenes* on refrigerated bologna. These researchers also reported that the combined treatment reduced the *L. monocytogenes* populations to below detectable levels by 8 weeks of refrigerated storage. The combined

effect of pediocin (3000 and 6000 AU) with post-packaging pasteurization (71, 81, 96°C) frankfurters during storage at 4, 10, and 25°C for 12 weeks resulted in no increase in *L. monocytogenes* populations up to 7 weeks for the combined treatment at any of the temperatures tested, however after 7 weeks, the counts increased for most samples (Chen et al., 2004). Survival data of *L. monocytogenes* from the studies mentioned above indicate that the survival and subsequent growth of *L. monocytogenes* on RTE meat surfaces would depend on various factors such as heating temperature, product characteristics, and the antimicrobial agents used.

CONCLUSIONS

Data on the levels of *L. monocytogenes* contamination during post-processing operations in the preparation of RTE meat products is limited. Epidemiologic data indicate that foods involved in listeriosis outbreaks are those in which the organism has multiplied and in general have contained levels significantly higher than100 cfu/g (Buchanan et al., 1997; ICMSF, 2002). Therefore controlling the growth of the organism during refrigerated storage is an important step in reducing the risk of listeriosis.

The main objective of this study was to evaluate the efficacy of surface application of nisin and/or lysozyme in combination with in-package pasteurization of RTE low fat turkey bologna against *L. monocytogenes* on the bologna surfaces during refrigerated storage for 3 months. Previous studies have demonstrated that a single intervention method is not adequate to control the growth of *L. monocytogenes* on RTE meat products during refrigerated storage. Results from this study indicate that combining in-package pasteurization of vacuum packaged RTE low fat turkey bologna at 65°C for 32 s with pre-surface application of nisin and nisin-lysozyme treatments significantly reduced a

relatively high initial inoculated population of *L. monocytogenes* and prevented the outgrowth during refrigerated storage. There were no *L. monocytogenes* cells detected after 3 weeks of storage for nisin and nisin-lysozyme treatments and no cells detected after 8 weeks for lysozyme treated bologna. The intervention methods used in the present study satisfies the requirements of alternative 1 of the interim final rule implemented by the USDA which requires the use of a post-lethality treatment to reduce the initial *L. monocytogenes* population and an antimicrobial agent or process to suppress or limit the growth of the pathogen during storage. Another advantage observed for combining pasteurization with antimicrobials was that the desirable antilisterial effects were obtained using lower levels of both treatments. This fact would help the industry in developing a cost-effective pasteurization method to control *L. monocytogenes* in RTE meat products and to preserve the desirable qualities of the food product.

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Fig. 5.1. Mean *Listeria monocytogenes* populations on pasteurized and non-pasteurized turkey bologna for various antimicrobial treatments during refrigerated storage. Each data point represents mean of 3 replications. 0.00 log cfu/cm^2 represents an undetectable (lowest detection limit 0.5 log cfu/cm^2) number of *Listeria* cells.