

NATURAL INHIBITION OF *LISTERIA MONOCYTOGENES* IN READY TO EAT MEAT
PRODUCTS

by

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(Under the Direction of Romeo T. Toledo)

ABSTRACT

Ready to eat meat products have been the source for the isolation of some LAB which are known to produce metabolic byproducts with inhibitory activity against spoilage and pathogenic bacteria. *Leuconostoc carnosum* 4010 was selected as the antimicrobial agent (cells and metabolic products) to be incorporated into an edible film. MIC was 1 ml of LAB cell-free supernatant against ca. 100 CFU/cm² of *Listeria*. Inhibitory activity was a synergistic effect of the bacteriocin and lactic acid. Viable LAB cells had an effect after 21 days at 4°C. The antimicrobial film C, 0.51 ± 0.02 mm thick, demonstrated the highest inhibitory action against *L. monocytogenes*. After 14 days of treatment, almost 2 log₁₀ CFU/25 cm² reductions were achieved from initial *Listeria* inoculation of 10³ CFU/25 cm². Antimicrobial film is a promising form of active food packaging as a response to the increasing demand for “natural” and “minimally processed” foods.

INDEX WORDS: *Listeria monocytogenes*, lactic acid bacteria, ready to eat meat and active food packaging.

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B.S., La Molina National Agrarian University, Perú, 2002

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment
of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2006

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May 2006

DEDICATION

To God who gave me life to live this wonderful experience. To my brother, Erik Mora, who gave me the opportunity to be better; his support, advice and encouragement help me to overcome all the difficulties and achieve my goals. To my parents, Edgar A. Mora and Lourdes A. Cossio, whose love and strong belief in me gave me the courage and strength to reach my dreams. To my extended family who helped me in different ways and to my special friends who shared with me wonderful memories.

This work is also dedicated to all my teachers who gave me guidance and encouragement.

ACKNOWLEDGEMENTS

I would like to express my gratitude and admiration to Dr. Romeo T. Toledo, who gave me guidance and support during the development of this project. To Dr. Mo Toledo, whose company and encouragement made my life even more enjoyable. My sincere and eternal gratitude to both of you.

I would like to thank Dr. Harrison for his patient and constant guidance and Dr. Hung for his support in being part of my committee.

I would also like to thank Revis Chmielewski and Ruth Ann Morrow for their help during the laboratory work and for their valuable advice. Thank you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Purpose of the study

Food safety has become an increasingly important concern in the food processing industry and *Listeria monocytogenes* is one of the most relevant emerging pathogens associated with foodborne illness. The cost of foodborne illness associated with the seven major pathogens has been estimated between \$6.5 and \$34.9 billion in the United States. These include: *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and *Toxoplasma gondii* (Buzby and Roberts 1997). The Centers for Disease Control and Prevention in the United States suggested that there are 76 million cases of foodborne illness in the U.S.A. each year, resulting in about 5000 deaths (Mead and others 1999).

Listeria monocytogenes emerged as a problem in deli meats and other processed meat products during 1980. Since then, outbreaks have been reported in 1998, 2000 and 2002. Hot dogs and deli (luncheon) meats were involved and the CDC reported 38 illnesses and 4 deaths for the 1998's outbreak; 29 cases, 4 deaths and 3 miscarriages for the 2000's outbreak and 46 cases, 7 deaths and 3 stillbirths for the one occurred in 2002 (CDC 1998; 2000; 2002). Consequently, FSIS implemented additional regulations where establishments producing deli meats and hot dogs should have a validated *Listeria* program; otherwise they were subject to an intensified FSIS testing program (USDA-FSIS 2004). By June 6, 2003 a final rule was

incorporated in the Code of Federal Regulations entitled as: “Control of *Listeria monocytogenes* in post-lethality exposed Ready-to-Eat Products” (9CFR430.4). The rule mandates that meat and poultry processors must follow one of the three alternatives proposed, where two of them call for the use of a growth inhibitor for *L. monocytogenes* in RTE products. All these rules were established to comply with the ‘zero tolerance’ policy for *Listeria monocytogenes* in this type of products.

Since then, outbreaks in RTE meat products haven’t been reported; however, contamination of the final products with *Listeria* have still been reported through the known recall process. Food recalls in the U.S. has increased during the last years due to the intensive food safety and security programs provided by the U.S. government. In 2004, the USDA/FSIS presented 13 recall cases of RTE meat products with possible contamination of *Listeria*; and in 2005 the cases reached 36. By January 5, 2006 one new case of meat product was recalled. *Listeria* contamination has produced negative economic impact on the meat industry. Consequently, many of the members of the meat and poultry industries now carry recall insurance. This last strategic response has had a positive effect on the industry promoting better food safety programs which reduces the costs on the company (Kramer and others 2005).

Increasing consumer dissatisfaction for formulated foods containing chemical preservatives has pushed the industry into the development of more “natural” and “minimally processed” foods (Cleveland and others 2001). This trend has led to the development of natural antimicrobial agents that can ensure the safety of the product without changing its preferred attributes of flavor and texture. The Code of Federal Regulations cites harmless lactic acid producing bacteria as protective agents in meat products; however no specific microorganisms are specified under this regulation (9CFR424.21). Lactic acid bacteria show special promise as a

protective culture due to inherent special properties described by many authors. Antimicrobial packaging is a promising form of active food packing, especially for RTE meat products, since contamination occurs mainly at the surface due to post-processing handling. On the other hand, environmental pollution has become another issue of concern during recent years and many researchers have focused on the development of biodegradable films, using mainly food protein, lipids or polysaccharides. Starch has been found as an alternative for edible films because it is biodegradable, abundant, cheap and easy to use (Mali and others 2002).

The purpose of this study was to evaluate three promising meat-borne lactic acid bacteria (LAB) in their ability to inhibit *L. monocytogenes* in a laboratory model system, incorporate the best one into an edible starch base film and finally, test it on a real food system. This research was focused on the potential use of a combination of viable LAB cells and their metabolic products in an edible film for better inhibition action as result of a hypothesized synergistic effect.

1.2 General characteristics of *Listeria monocytogenes*

Listeriae are Gram-positive, non-spore-forming, non-acid fast rods, catalase positive, and facultative anaerobes that were once classified as “*Listerella*” (Jay 2000a; Downes and others 2001). *Listeria monocytogenes* is also oxidase negative and expresses a β - hemolysin which produces zones of clearing on blood agar (Farber and Peterkin 1991). The organism was named *Listerella monocytogenes* after the mononucleosis-like illness was observed by Murray et al. in 1926 and Pirie documented the disease’s similarity to *Listerella hepatolytica* in 1927 (Murray 1926; Pirie 1927; Downes and others 2001). However, after learning that the generic name

Listerella had been previously used to describe a slime mold and a marine protozoan (Gibbons 1972), the name was change to *Listeria monocytogenes* in 1940 (Jay 2000b).

1.2.1 Taxonomy

For many years, the genus *Listeria* was monospecific, containing only the type species *L. monocytogenes*. However, the genus *Listeria* now contains seven species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and one environmental isolate closely related to *L. innocua* (Low and Donachie 1997; Wiedmann and others 1997). In many ways they are similar to the genus *Brochothrix* and closely related to *Bacillus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus* within the *Clostridium-Lactobacillus-Bacillus* branch. *Brochothrix* shares 338 common purine and pyrimidine bases with *Listeria*. Teichoic and lipoteichoic acids are found in *Listeria* spp., as in the bacilli, staphylococci, streptococci, and lactobacilli, but unlike these groups, their colonies form a blue green sheen when viewed by obliquely transmitted light (Jay 2000b). The six species of *Listeria* are characterized by the possession of antigen that gives rise to 17 serovars; the primary pathogenic species of *L. monocytogenes* are represented by 13 serovars. The 1998 – 1999 outbreak in the United States was caused by a rare strain of serovar 4b. In 1966, 60% of the isolates from human cases in the United Kingdom were 4b with 17%, 11% and 4% caused by 1/2a , 1/ab and 1/2c, respectively (Ternstrom and Molin 1987). In general, 4b strains are more often associated with outbreaks while 1/2 strains are associated with food products (Jay 2000b).

1.2.2 Growth characteristics

Listeria monocytogenes grows between -0.4 and 50°C (Junttila and others 1988). Inoculated broth cultures typically become turbid within 8 – 24h of incubation at 35°C. When *Listeria* grows on nutrient agar, the colonies are typically smooth, bluish gray and slightly raised

and measure 0.2 – 0.8mm in diameter after 24h of incubation (Downes and others 2001). Cultures on clear media exhibit a characteristic blue-green iridescence when examined with a binocular microscope under obliquely transmitted light (Gray 1956; Lachica 1990). The organism possesses peritrichous flagella, which give it tumbling motility, occurring only in a narrow temperature range. When the organism is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (Peel and others 1988).

Nutrient requirements

The nutritional requirements of *Listeria* spp. include at least four B vitamins: biotin, riboflavin, thiamine, and thioctic acid; and the amino acids cysteine, glutamine, isoleucine, leucine, and valine (Sneath and others 1986; Kim and Frank 1994). They grow well in many common media such as brain heart infusion, trypticase soy, and tryptose broths (Jay 2000b). Glucose enhances growth of all species, and during the growth, lactic acid and acetic acid is produced (Wilson and others 2002). *Listeria* spp. is able to hydrolyze esculin, and grow in the presence of 10% or 40% (w/v) bile, in about 10% NaCl, 0.025% thallous acetate, and 0.04% potassium tellurite; but they do not grow in the presence of 0.02% sodium azide. Although iron is important in its *in vivo* growth, *L. monocytogenes* apparently does not possess specific iron-binding compounds, and it obtains its needs through the reductive mobilization of free iron, which binds to surface receptors (Jay 2000b).

Effect of pH

Listeria grows best in the pH range 6 – 8. In general, some species will grow over the pH range of 4.1 to around 9.6 (Jay 2000b). However, the minimum growth pH is a function of temperature of incubation, overall nutrient composition of growth substrate, water activity and

the presence and quantity of NaCl and other salts or inhibitors. For example, growth at minimum pH is influenced by temperature in the following cases: pH 4.4 and 30°C growth was observed in less than 7 days, pH 4.4 and 20°C growth occurred in 14 days; and pH 5.23 at 4°C growth occurred in 21 days (George 1988; Jay 2000b).

The interaction of pH with NaCl and incubation temperature has been the subject of several studies (Conner and others 1986; Cole and others 1990). Some of the findings are for example: at pH 4.66, time to visible growth was 5 days at 30°C with no NaCl added, 8 days at 30°C with 4.0% NaCl, and 13 days at 30°C with 6.0% NaCl, all at the same pH (Cole and others 1990). The pH and NaCl effects were determined to be purely additive and not synergistic in any way (Jay 2000b).

Effect of temperature

The optimum growth temperature range is between 30 and 37°C. The mean minimum growth temperature of 78 strains of *L. monocytogenes* was found to be $1.1^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$, with a range of 0.5 – 3.0°C (Junttila and others 1988; Holt and others 1994). These investigators suggested that hemolysin may enhance growth and survival of *L. monocytogenes* in cold environments because this specie had about a 0.6°C lower minimum temperature than the other species of Listeria. The maximum growth temperature for listeriae is around 45°C (Jay 2000b).

Effect of water activity

Studies showed that *L. monocytogenes* is second only to the staphylococci as a foodborne pathogen in its ability to grow at a_w values < 0.93 (Jay 2000b). Using brain heart infusion (BHI) broth, three humectants, and 30°C incubation, the minimum a_w that permitted growth of serotypes 1, 3a, and 4b of *L. monocytogenes* was as follows: with glycerol 0.90, sucrose 0.93, and with NaCl 0.92 (Farber and others 1992).

1.2.3 Ecology

Listeria monocytogenes is an ubiquitous organism and has been isolated from a variety of sources including soil, mud, silage, decaying vegetation, water, sewage, and feces (Welshimer 1968; Dijkstra 1982; Al-Ghazali and Al-Azawi 1986; Fenlon 1986). However, its primary habitat seems to be in soil and vegetation where the bacterium leads a saprophytic existence (Welshimer 1960), with soil serving as a reservoir for infections transmitted later to animals and humans (Weis and Seeliger 1975).

Silage can support the growth of a diverse group of *Listeria* strains if it is improperly fermented or moldy with a pH > 5 (Fenlon 1986; Ryser and others 1997), and it has been a source of infection for cows, sheep and goats (Fensterbank and others 1984; Fenlon 1986). Numerous animal species are susceptible to listerial infections, with many healthy animals cited as asymptomatic fecal carriers of *L. monocytogenes* (Ryser and Donnelly 2001). In humans, fecal carriage rates have reportedly varied from 0 to 77% depending on the extent of exposure, with approximately 5% of the general population assumed to be asymptomatic shedders of *L. monocytogenes* (Low and Donachie 1997).

1.2.4 Virulence properties

Of listerial species, *L. monocytogenes* is the pathogen of human concern. The ingestion of viable cells is necessary for *Listeria* infection to occur. *Listeria monocytogenes* is an intracellular pathogen whose most significant virulence factor is listeriolysin O (LLO), which is an exocellular thiol-activated, pore-forming substance that has been detected in all strains of *L. monocytogenes*; however it does not per se cause the foodborne gastroenteritis syndrome. LLO is a hemolysin involved in the invasion of the gut epithelium and it contributes to the cell-to-cell spread of the organism. LLO is a substance responsible for beta-hemolysis on erythrocytes and

the destruction of phagocytic cells that engulf them. *Listeria monocytogenes* can breach the mucous barrier and enter epithelial cells, but just how is unclear (Jay 2000b). Gastrointestinal symptoms are seen in only around one third of human cases (Gellin and Broome 1989).

The organism crosses the mucosal barrier of the intestine and invades the bloodstream. It may disseminate to any organ, but it has a clear predilection for the placenta and the Central Nervous System (CNS), thereby determining the main clinical syndromes (Siegman-Igra and others 2002). As an intracellular pathogen, it must first enter susceptible cells. In the case of phagocytes, entry occurs in two steps: directly into phagosomes and from the phagosomes into the phagocyte's cytoplasm. Entry into nonphagocytic cells is different. Uptake requires surface-bound proteins of the bacterium designated InlA and InlB (Lingnau and others 1995). The InlA protein is required for entry into cultured epithelial cells, whereas InlB is required for invasion of cultured mouse hepatocytes (Drevets and others 1995). *Listeria monocytogenes* survives inside macrophages by escaping from phagolysosomal membranes into the cytoplasm (cytosol), and this process is facilitated in part by LLO (Jay 2000b). Once inside the cytosol, the surface protein ActA aids in the formation of actin tails that propel the organism toward the cytoplasmic membrane. At the membrane, double membrane vacuoles form. With LLO and two bacterial phospholipases, the bacteria are freed and the process is repeated upon entry of bacteria into adjacent host cells. The latter occurs following the pushing out of the membrane to form a filopodium (a projection), which is absorbed by an adjacent cell and the invasion process is repeated. Thus, the spread of *L. monocytogenes* from cell to cell occurs without the bacterium having to leave the inner parts of the host cells (Jay 2000b).

1.2.5 Disease syndrome

Listeriosis in humans is most often seen as an invasive illness in certain well-defined high-risk groups, including immunocompromised adults, pregnant women and neonates, but may also occasionally occur in individuals with no predisposing conditions (Slutsker and Schuchat 1999). Unlike most other foodborne illnesses, listeriosis may take several months to develop after initial exposure and exhibit a mortality rate of 20% to 30% of those who become ill (Mead and others 1999). Bacteremia, in the U.S., was the most common manifestation of listeriosis in non-pregnant adults, followed by meningitis and meningoencephalitis. Patients with bacteremia most often experience fever, malaise, fatigue, and abdominal pain, while those individuals with central nervous system involvement develop fever, malaise, ataxia, seizures and altered mental status (Ryser and Donnelly 2001). Among pregnant women, listeriosis is typically reported during the third semester exhibiting only a mild flu-like illness. However, intrauterine infections can result in premature delivery, spontaneous abortion, stillbirth, or early-onset neonatal listeriosis (Lorber 1997). A late-onset form of neonatal listeriosis can also be developed several weeks after birth as a result of infection during delivery; this is characterized by a highly fatal form of meningitis (Gellin and others 1991). The mother is rarely severely affected by listeriosis as the disease appears to focus on the fetus (Rocourt 1996). In healthy non-pregnant adults, *L. monocytogenes* can produce a mild, self-limiting non-invasive form of gastrointestinal illness, characterized by the development of fever, diarrhea, headaches and myalgia within 12 to 24 hours of exposure (Dalton and others 1997).

1.2.6 Infectious dose

There are many studies with mouse models that confirm the greater susceptibility to *L. monocytogenes* of animals with impaired immune systems than normal animals, as is the case with humans. The correspondence of minimal infectious doses for normal adult mice to humans is more difficult. It has been suggested that levels of *L. monocytogenes* less than 10^2 cfu/g appear to be inconsequential to healthy hosts (Golnazarian and others 1989). However, the consumption of higher doses (10^6 cfu/g food) for healthy individuals can cause illness, but only 10^2 cells/g may be required for immuno-compromised individuals (Low and Donachie 1997). Contaminated foods generally contains low levels of *Listeria* between 50 to 100 cfu/g (Besse and others 2005); however, once food products are contaminated post-processing, subsequent refrigeration or frozen storage will have poor effect on decreasing the population of *L. monocytogenes*, and after few hours or days, depending on temperature and time, it can reach higher numbers able to produce sickness even on healthy individuals.

1.3 Lactic acid bacteria

Fermented foods have been consumed during the 6000 to 12000 years of man's cultural history. By trial and error, man has devised methods of "controlled" fermentation in order to counteract undesired deterioration of products of plant and animal origin. Beneficial microorganisms were favored by selected parameters while spoilage and other deleterious microbes were inhibited (Holzapfel and others 1995). The earlier fermentation products started with fermented beverages such as beer and wine, and also milk products like cheese, yogurt, etc. By the 9th century BC, developments included the fermentation of meat. By the 3rd century BC, vegetables were added to the list (Pederson 1971).

Protective cultures have a primary effect in preserving fermented products because of their antimicrobial action and should be considered as an additional safety factor supporting good manufacturing practices (GMP) and a validated plant's Hazard Analysis Critical Control Point (HACCP). In addition, under abuse conditions of temperature, handling, etc. their metabolic activities (e.g. acid or gas production) may serve as an indicator of microbial risk (Holzapfel and others 1995).

The lactic acid bacteria (LAB), generally considered as 'food-grade' organisms, show special promise for selection and implementation as protective cultures (Holzapfel and others 1995). Members of the genus *Lactobacillus* are most commonly given "safe" or "generally recognized as safe" (GRAS) status, whereas members of the genera *Streptococcus* and *Enterococcus* contain many opportunistic pathogens (Donohue and others 1993) that in rare cases were involved in human infections (Holzapfel and others 1995).

1.3.1 Microbiology

The lactic acid bacteria (LAB) are all Gram-positive; anaerobic; micro-aerophilic or aerotolerant; catalase negative; nonsporulating cocci or rods. They all produce lactic acid as the sole, major or an important product from the energy-yielding fermentation of sugars (Wood and Holzapfel 1992). Some also produce volatile acids and CO₂ (Hall and others 2001).

1.3.2 Taxonomy

Historically the core of LAB comprised four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Currently, taxonomists generally consider the following genera to comprise this group: *Aurococcus*, *Alliococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. The

classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. For some of the newly described genera, additional characteristics such as fatty acid composition and motility are used in classification (Axelsson 1998).

1.3.3 Antimicrobial components – Mode of action

Historically, LAB have been used to preserve food due to the antimicrobial substances they produce. Homofermentation produces lactic acid while heterofermentation produces equimolar amounts of lactic acid, acetic acid/ethanol, and carbon dioxide (Axelsson 1998). Some strains are also able to produce bacteriocins and few strains produce specific antimicrobial substances like reuterin and pyroglutamic acid (Ouweland 1998).

Organic acids

LAB metabolism produces mainly two acids: lactic acid and acetic acid. Of the two acids, acetic acid is the strongest inhibitor and has a wide range of inhibitory activity, inhibiting yeast, mold and bacteria (Blom and Mörtvedt 1991). This can be explained by the higher dissociation constant (pK_a) of acetic acid as compared to lactic acid (4.75 and 3.08, respectively) (Holzapfel and others 1995). For example, at pH 4, only 11% of the lactic acid is undissociated whereas 85% of the acetic acid is undissociated (Eklund 1983; Ouweland 1998). Acetic acid is usually present in small concentrations; however, it may constitute a vital factor for the establishment of the initial LAB population (Buckenhüskes and others 1990). The undissociated (neutral) form of the organic acid diffuses across the cell membrane because it is lipid-soluble, thereby reducing the intracellular pH and slowing down the metabolic activities responsible for growth inhibition (Cramer and Prestegar 1977).

Hydrogen peroxide

LAB are able to generate hydrogen peroxide (H_2O_2) in the presence of oxygen together with lactate, pyruvate and NADH by flavin enzymes (Kandler 1983). The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell; sulfhydryl groups of cell proteins and membrane lipids can be oxidized (Lindgren and Dobrogosz 1990). The main antimicrobial effect is attributed to blocking of glycolysis due to oxidation of sulfhydryl groups in the metabolic enzymes. Glyceraldehyde-3-phosphate dehydrogenase appears to be the primary target (Carlsson and others 1983). The effect against Gram positive bacteria is generally bacteriostatic, whereas it is bactericidal for many Gram negative bacteria (Blom and Mörtvedt 1991).

Low molecular weight metabolites

Carbon dioxide: Heterofermentative LAB mainly produce carbon dioxide (CO_2) during the fermentation of hexoses. Carbon dioxide has a dual antimicrobial effect because its formation creates an anaerobic environment and carbon dioxide in itself has an antimicrobial activity. The precise mechanism of action is unknown but it has been suggested that the accumulation of carbon dioxide in the lipid bilayer causes dysfunction in permeability (Lindgren and Dobrogosz 1990).

Diacetyl is produced by some *Lactococcus*, *Leuconostoc* and *Pediococcus* spp.; however, this component has little potential in the preservation of food due to its intense aroma (Holzapfel and others 1995).

Reuterin is produced by *Lactobacillus reuteri* in the presence of glycerol and coenzyme B12. It has a broad-spectrum antimicrobial activity and has been suggested for biopreservation of fish and meat (Lindgren and Dobrogosz 1990).

Pyroglutamic acid was found to be produced by *Lactobacillus casei* ssp. *casei*, *L. casei* ssp. *pseudoplatanturum* and *Streptococcus bovis* (Huttunen and others 1995). It was found to be inhibitory to *Bacillus subtilis*, *Enterobacter colacae* and *Pseudomonas putida* (Ouwehand 1998).

Bacteriocins

Bacteriocins are defined as a group of potent antimicrobial peptides or proteins produced by bacteria and mainly kill or inhibit the growth of closely related species by adsorption to receptors on the target cells. Bacteriocins are ‘secondary’ metabolites ribosomally produced (Holzapfel and others 1995). Some LAB found in fermented and non-fermented foods produce a high diversity of bacteriocins. However, nisin is currently the only bacteriocin approved for use in the U. S. and throughout the world as a food preservative (Cleveland and others 2001).

Bacteriocins have been divided in three or four groups (Klaenhammer 1993; Nes and others 1996). Class I is subdivided in subclasses: Class Ia and Class Ib. Class I is represented by peptides with 19 to more than 50 amino acids, and they are characterized by their unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Class Ia consists of cationic and hydrophobic peptides that form pores in target membranes and present a flexible structure compared with the more rigid Class Ib. Class Ib bacteriocins are globular peptides and they have no net charge nor a net negative charge (Altena and others 2000). Class II consists of small (<10kDa) heat-stable (100 to 121°C), non-modified peptides. Class II is further subdivided in Class IIa and IIb. Class IIa includes Pediocin-like *Listeria* active peptides. Class IIb consists of bacteriocins composed of two different peptides, and both peptides are needed to be completely active. There was a Class IIc originally proposed for bacteriocins that are secreted by the general secretory system of the cell (Nes and others 1996); however it has been proposed that this Class IIc should be eradicated since the Class IIa bacteriocins have been shown to be

produced by the secretory system (Cintas and others 1997). On the other hand, Ouwehand (1998) considers Class IIc bacteriocins those that are thiol-activated peptides. Class III bacteriocins are represented by large (>30kDa) and heat labile bacteriocins for which there is much less information available. The Class IV consists of large complex bacteriocins (protein with lipid and/or carbohydrates) (Klaenhammer 1993). However, there is a good reason to believe that these bacteriocins are just the result of a complex union between macromolecules and bacteriocins from the other classes due to their cationic and hydrophobic properties (Cleveland and others 2001). An example of this is plantaricin S first claimed as a large complex molecule but after purification was found to be a small peptide with the same activity as the complex peptide (Jimenez-Diaz and others 1995). Class I and II bacteriocins are the bacteriocin most understood and therefore holds more promise for food applications (Cleveland and others 2001). For most bacteriocins, the antimicrobial effect seems to be bactericidal (Schillinger 1990); however some seem to be bacteriostatic like leucosin S (Lewus and others 1992) and leucocin A-UAL 187 (Hastings and others 1991).

The primary target of class I bacteriocins, particularly lantibiotics, is the cell membrane. The model-type lantibiotic nisin has been studied as an example of the mode of action of this class of bacteriocins. Lantibiotics don't need other receptors as other peptides do, however it needs the presence of a transmembrane potential and/or pH gradient to form pores in the membrane resulting in inhibition of the target cell by leakage of cellular materials. There is a cation-exchange process where the strongly cationic lantibiotics displace autolytic enzymes that weaken the cell wall. The lantibiotics interfere with the cell's energy supply, inhibiting cell wall repair. The pores formed do not allow passage of high molecular weight compounds. This

causes an influx of water because of the increased osmotic pressure resulting in cell lysis (Sahl and others 1995).

Class II bacteriocins have also been shown to be membrane active peptides, destroying the membrane by the formation of pores. However, these bacteriocins act on the target cells regardless of their energization (Jack and others 1995). Lactococcins were studied as a model for this class of bacteriocins. The studies suggested that lactococcin A could form a membrane-spanning α helix with amphiphilic properties. The aggregation of the molecules in the manner of barrel staves around a central water-filled pore produced the pore formation and the size of the pore depend on the bacteriocin concentration (Klaenhammer 1993; Venema and others 1995). The formation of pores explains the increase of membrane permeability and dissipation of the proton motive force (Bruno and Montville 1993; Jack and others 1995). Other Class II bacteriocins have been found to act by inhibiting RNA, DNA, protein synthesis, transport of precursors and dissipation of the proton motive force in the cell wall target (Bruno and Montville 1993; Venema and others 1995; Gonzáles and others 1996).

1.4 Regulatory background

Listeria monocytogenes became a problem in processed meat and poultry products during 1980's. Outbreaks have been reported in 1998, 2000 and 2002. Hot dogs and deli (luncheon) meats were involved and the CDC reported 38 illnesses and 4 deaths for the 1998's outbreak; 29 cases, 4 deaths and 3 miscarriages for the 2000's outbreak and 46 cases, 7 deaths and 3 stillbirths for the one that occurred in 2002 (CDC 1998, 2000, 2002). As a response, FSIS published a notice advising RTE meat and poultry processors to reassess their HACCP plans to ensure the problem of *L. monocytogenes* is correctly addressed (USDA-FSIS 2004).

A new outbreak during the summer of 2002 led FSIS to conclude that some processors were not adequately addressing the potential for bacterial contamination in their HACCP plans, SSOP's and other control measures. Consequently, in December 2002 FSIS implemented additional steps to be taken by the USDA inspectors. Under 9CFR417.4 establishments producing deli meats and hot dogs should have a validated *Listeria* program; otherwise they were subject to an intensified FSIS testing program that includes increased product and food contact surface testing, environmental testing in the plant, and increased reviews of plant records and data (USDA-FSIS 2004).

By June 6, 2003 the USDA and FSIS issued the final rule 68 FR 34224 incorporated in the Code of Federal Regulations entitled: "Control of *Listeria monocytogenes* in post-lethality exposed Ready-to-Eat Products" (9CFR430.4). The rule mandates that meat and poultry processors must follow one of the three alternatives proposed in order to control *Listeria monocytogenes* in RTE products. The first alternative calls for the application of both post-lethality treatment and a growth inhibitor for *Listeria* on RTE products, the second alternative calls for the use of one of them, and the third one calls for the application of sanitation measures only. The intensity of verification testing by FSIS is considered higher if the establishment chooses Alternative 3 in relation with Alternative 2 or 1. All these rules were established in order to comply with the regulatory policy of 'zero tolerance' of *Listeria monocytogenes* in RTE products. Consequently, the food industry faces a need for natural antimicrobial agents that can ensure the safety of the product without changing the product's preferred attributes of flavor and texture.

The Code of Federal Regulations cites harmless lactic acid producing bacteria as protector agents in meat products; however, no specific microorganisms are mentioned under

this regulation (9CFR424.21). To date, nisin is the only bacteriocin that has been accepted by the USDA-FSIS as GRAS (Generally Recognized as Safe) (21CFR184.1538). Many other bacteriocins from lactic acid bacteria have been discovered, characterized and studied extensively in order to offer more alternatives to the food industry; however one hurdle that needs to be overcome before its commercial use as a biopreservative is its legal acceptance as a food additive.

Lactic acid bacteria show special promise as a protective culture. The ideal LAB should present the following properties: no health risks, competitiveness against undesired microorganisms, no negative (sensory) effects on the product under GMP and function as 'indicator' under abuse conditions (Holzapfel and others 1995).

1.5 Ready-to-eat meat and poultry products

Cooked uncured products: The quality of the raw materials and the cooking process are the starting points to determine the final microbiology of the cooked meats. During the cooking process the temperatures are sufficiently high to destroy all the microorganisms in a way that only spores survive. However, the internal microbial level will depend also on the subsequent holding time and temperature. The normal plate count at 35°C of freshly prepared uncured meats is 10^2 or less per gram. RTE meats and poultry products tend to be further contaminated on the surface from equipment and food handlers during subsequent handling, packing, or serving (Tompkin and others 2001).

Microbial growth is very favorable in cooked uncured meat products due to their highly nutritious medium, favorable pH and low salt content. Considering this, most regulations prohibit keeping precooked meats between 5 and 60°C except during preparation, heating or

chilling. Most of these products are frozen for shipping and distribution. The type of spoilage flora will depend on factors such as packaging and temperature. Some food products that fall into this category had caused foodborne illness because of a failure to observe good practices in preparing, holding, and serving in homes, restaurants, and institutions (Varnam and Sutherland 1995; Tompkin and others 2001).

L. monocytogenes has been isolated from a wide range of cooked meats, including roast beef and ham. Some evidence indicates that the contamination has been derived from environmental sources rather than from the raw meat. In addition, plant colonization by the organism may also be involved (Varnam and Sutherland 1995).

Cooked cured products: This category include ham, bologna, franks, and a wide variety of luncheon meats made from red and /or poultry meat. The heating step eliminates most of the microorganisms with exception of spores and thermotolerant bacteria. During preparation of these products, contamination tends to occur on the surface. Processors have been using salt, nitrite, and others permitted antimicrobial agents like sodium lactate, sodium diacetate, potassium lactate and trisodium phosphate (9CFR424.21). After packing, the normal plate count of these products is 10^3 or less per gram. Higher levels will reflect the time-temperature history of the product. Luncheon meats are usually packaged under vacuum or in a modified atmosphere presenting very low oxygen content. Lactic acid bacteria is the dominant microflora in commercially packaged cured meats (Tompkin and others 2001). Coliforms are unavoidable contaminants at low levels ($\pm 10^1$ per gram); *S. aureus* do not represent a risk in luncheon meats because it cannot grow in anaerobic conditions, presence of nitrate and salt nor refrigeration storage of these type of products. On the other hand, ham is frequently implicated in outbreaks of staphylococcal foodborne illness due to contamination by food handlers. For example, during

slicing and then subsequent holding time at warm temperatures for enough time to produce the enterotoxin (Tompkin and others 2001).

Contamination with *L. monocytogenes* has occurred frequently in these type of products throughout the world; however few outbreaks have been reported. In North America only two cases of human listeriosis have been linked with the consumption of these type of products (Tompkin and others 2001).

1.6 Lactic acid bacteria in RTE meat products and their competitive inhibition toward *L. monocytogenes*

Many studies have been done in order to identify the dominant lactic acid bacteria in cooked meat products. The LAB strains generally considered natural microflora in meats and meat products are: *Carnobacterium piscicola* and *C. divergens*; *Lactobacillus sakei*, *Lb. curvatus* and *Lb. plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc gelidum* and *Leuconostoc carnosum* (Hugas 1998).

Vermeiren and others (2004) screened and characterized 91 strains of LAB originating from meat products for potential protective cultures to be used in the cooked cured meat industry. The strains were tested for the antibacterial properties and 91% of the strains could inhibit *L. monocytogenes*. Finally, 12 strains were identified with the highest antibacterial capacities and those were evaluated by their growth rate, acidifying character and lactic acid production at 7°C under anaerobic conditions. Two strains were bacteriocin producing and those were identified as *Lactobacillus plantarum* and *Lactobacillus sakei* 148, with the former being the slowest growing strain. Cooked ham was inoculated with strains identified as *L. sakei* subsp. *carnosus*, *L. sakei* 148 and *L. sakei* subsp. *carnosus* SAGA 777. The inoculated samples were

not rejected by the sensory panel after 34 days of storage under vacuum packaging at 7°C. Greco and others (2004) studied the LAB in a typical Italian dry fermented sausage. The LAB was dominant during ripening and mainly consisted of homofermentative mesophilic rods. The main isolates were identified as *Lactobacillus sakei* (43.3%), *Lactobacillus plantarum* (16.6%) and *Lactobacillus curvatus* (13.3%). In 2002, De Martinis and Freitas screened twenty samples of vacuum-packed meat products from Brazil for the presence of bacteriocin-producing LAB, using the agar overlay method. The LAB identified were *Enterococcus* sp. 18 (from bacon), *Leuconostoc* sp. 20 (from ham) and *Lactobacillus sakei* 29 (from home made “linguica”). *Leuconostoc* sp. 20 and *Lactobacillus sakei* 29 presented antilisterial activity against all the strains of *L. monocytogenes* tested. According to Vermeiren and others (2004), after the analysis of 27 different cooked meat products, the main isolate was *Lactobacillus sakei* (47.6%). Other isolates were *Lactococcus lactis* subsp. *lactis* (23.8%), *Carnobacterium divergens* (14.3%), *Lactobacillus curvatus* (9.5%) and *Lactobacillus acidophilus* (4.76%). On the other hand, fermented meat products contained more of *Lactococcus lactis* subsp. *lactis* (25.0%), *Lactobacillus curvatus* (18.8%), *Carnobacterium divergens* (14.3%), *Lactobacillus sakei* (12.5%), *Lactobacillus plantarum* (12.5%) and in a lower percentage: *Pediococcus pentosaceus*, *Leuconostoc lactis*, *Lactobacillus brevis* and *Leuconostoc mesenteroides* subsp. *mesenteroides*. Under this same study, *Lactobacillus plantarum* 5 was shown to produce bigger inhibition zones compared with 11 other selected strains of LAB identified as *L. sakei* and *L. curvatus*, when they were tested against three different *L. monocytogenes* strains.

Lash and others (2005) tested *Lactobacillus plantarum* ATCC 8014 for the production of antimicrobial compounds. The study showed the effect of a cell-free supernatant of *L. plantarum* toward the inhibition of Gram-positive and Gram-negative bacteria like *Listeria innocua*,

Staphylococcus aureus, *Escherichia coli*, and *Pseudomonas aeruginosa*. The bacteriocins were digested with different proteases in order to demonstrate their protein nature. The effect of temperature on the cell free supernatant was also tested. Water baths were adjusted at different temperatures of 24°C (control), 30°C, 50°C, 72°C, and 100°C and the cell free supernatant was submerged for 30 minutes. There was a loss of activity when the temperature was greater than 30°C. The pH effect on the antibacterial product(s) of *L. plantarum* was tested. Adjustments of pH were made using 1 N NaOH or 1 N HCl. As a result there was loss of activity of the antibacterial products when pH was lowered below 4 or above 5. This study supports the idea that the Gram-positive *L. plantarum* produces bacteriocins. All these findings present *L. plantarum* as a good candidate to be used for biopreservation of fermented meat products.

Budde and others (2003) studied the natural LAB present in vacuum-packed meat products, products such as ham, salami, cooked loin, and smoked bacon. These products were screened for bacteria with antibacterial properties; especially the ones with the ability to produce bacteriocins. Forty eight vacuum-packed meat products were analyzed and approximately 72,000 colonies were isolated. *Leuconostoc carnosum* was the predominant strain and *Leuconostoc carnosum* 4010 showed strong antilisterial activity without the production of any undesirable flavor in the meat product. Further studies were made and the bacteriocins identified were very similar to leucocin A and leucocin C. Also, the experiments showed that a concentration of 10^7 cfu/g of *Leuconostoc carnosum* 4010 applied to a vacuum-packaged meat sausage produced immediate reduction of *L. monocytogenes* to an undetectable level during storage at 5°C for 21 days. In a follow-up study of this new protective culture, Hornbæk and others (2004) examined the effect of sodium chloride and sodium nitrite in the inhibitory activity of *Leuconostoc carnosum* 4010 and its bacteriocins toward *Listeria monocytogenes* 4140. A gelatin system was

used as a solid matrix. Both components, sodium chloride (2.5% w/v) and sodium nitrite (60ml/l), reduced the inhibitory effect of *L. carnosum* 4010 and its bacteriocins. The lag phase of *L. monocytogenes* in the presence of *Leuconostoc carnosum* 4010 was 71 hours; the addition of sodium chloride and sodium nitrite reduced the lag phase to 58 h and 40 h, respectively during experiments at 10°C. Another discovery was that the maximum specific growth rate of *L. monocytogenes* 4140 in presence of *L. carnosum* 4010 was increased from 0.02 to 0.06 h⁻¹ by the addition of sodium chloride while sodium nitrite didn't produce any change. In 2003, Jacobsen and others found that live cells of *L. carnosum* 4010 added to the surface of the final product was more effective in preventing growth of *L. monocytogenes* than the use of the partially purified bacteriocins added before heat treatment to the fermented sausage (saveloy). *Leuconostoc carnosum* 4010 was added at a concentration of 10⁷ cfu/g, and *L. monocytogenes* was inoculated at 10 cfu/g approx. The experiments were performed at 5 and 10°C for 4 weeks. *L. monocytogenes* counts were below 10 cfu/g during the 4 weeks of storage at 10°C. The results showed that *L. carnosum* 4010 was a suitable protective culture for vacuum-packed meat products.

Many studies have been published about the effectiveness of bacteriocin producing LAB against *L. monocytogenes*; however, inhibitory effects has also been observed by non-bacteriocin producing LAB. In 2005, Vermeiren and others (2005b) focused their efforts in the evaluation of two LAB on a model of cooked ham. The non-bacteriocinogenic *Lactobacillus sakei* subsp. *carnosus* (10A) isolated from smoked turkey and the bacteriocin producing *Lactobacillus sakei* 148 (LS5) isolated from Spanish dry sausage, were tested for inhibition of three strains of *L. monocytogenes*. The results showed that only *L. sakei* 10A produced antagonistic effect toward *L. monocytogenes*; and the complete inhibition of *Listeria* was achieved in the presence of

combined strain 10A and a temperature of 4°C or a Modified Atmosphere package (MAP) containing 50% CO₂. The inoculum level was 10⁶ cfu/g for LAB 10A and 10³cfu/g for *Listeria*. The sensory evaluation showed that 10A at a high level did not acidify the product to a point of rejection. Also Amezcuita and Brashears (2002) found two LAB on cooked ham and frankfurters, *Lactobacillus casei* and *Lactobacillus paracasei*, with antilisterial properties. Bredholt and others (1999) identified five strains of *L. sakei* in cooked, sliced, vacuum- or gas-packed ham and sausage in Norway; the strains produced inhibition of *L. monocytogenes* and *E. coli* O157:H7. One of the possible explanations for the inhibition include the high production of lactic acid. However, in vacuum-packed cook meat products, the pH decline is not large enough to be considered the main reason due to the low glucose content and the buffering capacity of cooked meat products (Vermeiren and others 2005b). Another reason could be the direct competition for nutrients (Buchanan and Bagi 1997). A recent study (Nilsson and others, 2005) concluded that reduction of *Listeria* growth in the presence of a non-bacteriocin LAB *Carnobacterium piscicola* was partially achieved by glucose depletion. A clear explanation of the mode of action of the non-bacteriocin LAB has not been offered yet. However, all these studies have demonstrated the effectiveness and suitability of non-bacteriocin and bacteriocin producing LAB as a protective culture in ready to eat meat products.

Other studies have also demonstrated the influence of the packaging type in the effectiveness of the protective culture against *L. monocytogenes*. In a package under vacuum, *Listeria* showed a 4 log₁₀ increase during a 42 day storage period, while under MAP no considerable increase was observed. When LAB was included in combination with MAP, the presence of *L. monocytogenes* was completely prevented (Vermeiren and others 2005a). The packaging type had little or no effect on the LAB because it has been demonstrated that LAB,

specially *L. sakei*, are less sensitive to modified atmospheres containing CO₂ than *L. monocytogenes* (Devleighere and others 2001). The level of CO₂ in the MAP commonly used has been from 30 to 50% (Vermeiren and others 2005a); studies with MAP with only 30% CO₂ showed that the growth of *L. monocytogenes* was similar to the growth on vacuum-packed products (Beumer and others 1996). Protective culture (LAB) in combination with another hurdle technology achieved better results.

1.7 Routes of *Listeria* contamination in food processing facilities

Listeria monocytogenes is a foodborne pathogen of particular concern in ready-to-eat (RTE) meat products due to the high mortality rate in susceptible individuals (30%). *Listeria* has a ubiquitous character and also has the ability to resist unfavorable environmental conditions. Because of these characteristics, many studies have been done to find the possible routes by which *Listeria* gets introduced in the processing plant environment. After the implementation of the pathogen reduction rule (Hazard Analysis and Critical Control Point) of the U.S. Department of Agriculture (final rule 68 FR 34207), the presence of *Listeria* in raw materials decreased to less than 100 cfu/g (Jay 1999). *Listeria monocytogenes* should be absent in cooked meat products because the heating temperature for these products is at least 70°C for 30 min. (Samelis and Metaxopoulos 1999). However, the fully cooked meats may be contaminated with *L. monocytogenes* after the lethal heat treatment but before they are packaged and according to the USDA/FSIS these products are considered to be adulterated.

Listeria counts in meat products can increase in large numbers during the passage from the slaughter to the processing plant (Cox and others 1997). Berrang and others (2005) suggested that the raw products may be one source of *Listeria* contamination in the plant environment

because three of the four strains of *Listeria* identified to be resident in the plant were detected on raw product at some point during the study. These authors detected *L. monocytogenes* in all the raw products, once in the fully cooked products but never on the surfaces of the cooked product.

Samelis and Metaxopoulos (1999) mentioned the importance of the cooking temperature in *L. monocytogenes* inactivation in cooked meats. The study found that *Listeria* survived in tumbled meats cooked in boiling water to a core temperature below 70°C and in country style sausages heated to 65 to 68°C for 3-5 h. On the other hand, *Listeria* was killed in oven cooked tumbled meats heated to 72–75°C for 1-5 h depending on the product size. This study stated that survivors of the heating process appeared to be the main cause of post-process contamination. Uyttendaele and others (1999) found higher incidence rates of *Listeria* in whole cooked meat products after slicing (6.65%) than before slicing (1.56%) indicating cross-contamination in the slicing room. This study also suggested that multiple handling and processing steps increased the incidence of *Listeria* in the final product; 6.14% in cooked minced meat products and 3.96% in whole muscle cooked meat products. Gibbons and others (2006) indicated that *Listeria* can form biofilms on different surfaces. In this condition *Listeria* is more resistant to detergents and disinfectants and the survivors become a source for further contamination of other finished products. Due to the formation of biofilms (Lunden and others 2003) some *Listeria* strains become residents of the plant and eventually are detected in fully cooked products (Berrang and others 2005). Efforts to keep strict cleaning processes and validated HACCP plans reduce considerably the possible contamination with *Listeria*; however, recent USDA reports on recall notifications show sporadic presence of the pathogen in RTE meat products.

1.8 Recalls in the meat and poultry industry

During the past years, the number of food recalls in the U.S. has increased considerably due to the intensive food safety and security programs provided by the U.S. government. The recalls affecting the meat and poultry industry have increased from 38 in 1993 to 128 in 2002. The majority of recalls were due to *L. monocytogenes* and *E. coli* O157:H7; however, the incidence rate of these pathogens has decreased (Kramer and others 2005). In the specific case of *L. monocytogenes* in RTE meats, the incidence rate was between 0 to 50% (Jay 1996), and in recent years it has dramatically decreased from 3% in 1995 to 0.75% in 2003 (Kramer and others 2005). In 2004, the USDA/FSIS reported 13 recall cases of RTE meat products with possible contamination of *Listeria*. In 2005 the cases reached 36 and nine of those recalls were still active by January 13, 2006. USDA/FSIS reported 1 new case of meat product recall in Jan. 5, 2006 (USDA-FSIS 2005; 2006).

Studies have shown that the negative direct economic impact of recalls in the meat and poultry industry is not just due to the recalled product but also for the negative effect on the public perception moving the demand temporary toward non-meat products (Marsh and others 2004). Other studies indicate that recalls had been perceived by the public as a lax attitude of the industries toward quality control and food safety (Skees and others 2001; Marsh and others 2004). Consequently, many of the members of the meat and poultry industries now carry recall insurance. This last strategic response have had a positive effect on the industry's promotion of better food safety programs in order to reduce the costs to the company (Kramer and others 2005).

1.9 Improvements in food safety – Antimicrobial food packaging

The presence of *L. monocytogenes* in RTE products has become a major concern for the meat industry especially because contamination can occur on the surface of the product following processing. The number of recalls has increased recently and with that the economic losses for the industry (Kramer and others 2005). Consequently, there is actual necessity for innovative ways to increase the safety and shelf life of RTE meat products.

The meat processing industry in the United States relies strongly on packaging techniques like vacuum-packaging and Modified Atmosphere packaging (MAP). Almost 90% of the red meat produced in the U.S. is vacuum-packaged (Siragusa and others 1999). These techniques together with refrigeration have been important to preserve products from microbial spoilage; however the loss of meat products is still very significant. Studies have shown that some lactic acid bacteria produce metabolic components (organic acids, bacteriocins) able to inhibit closely related bacteria responsible for deterioration. However, the mode of their application in meat systems is critical for success.

Some studies have shown that the application of antibacterial agents into meat formulations may result in partial inactivation of the active substances due to interactions with the meat constituents and also due to processing effects like denaturalization of the bacteriocins by high temperatures. Consequently only a limited amount of antimicrobial agents are effective on the surface flora (Ouattara and others 2000b). Application of antimicrobial agents only on the meat surface by dipping or spraying have been shown to have the active agent rapidly diffuse into the bulk of the meat. Therefore the results on surface microflora inhibition were not completely satisfactory (Siragusa and Dickson 1992; Quintavalla and Vicini 2002). However slow diffusion of the antimicrobial agents away from the surface helps to maintain high

concentrations where they are needed (Ouattara and others 2000a). Packaging films containing antimicrobials have proven to achieve this purpose. Examples are: low density polyethylene (LDPE) films incorporated with imizalil or benzoic anhydride (Weng and Hotchkiss 1992; 1993), cellulosic solution containing nisin (Cha and others 2002), and nisin on meat surfaces (Siragusa and others 1999).

On the other hand, environmental pollution has become another issue of concern during recent years. Many researchers have focused on the development of biodegradable films, using mainly food proteins, lipids or polysaccharides. Recent studies have used edible coatings to test antimicrobial agents like sorbic acid, propionic acid, potassium sorbate, benzoic acid, sodium benzoate and citric acid (Ouattara and others 2000a; Quintavalla and Vicini 2002). Bacteriocins like nisin and pediocin (Sebti and Coma 2002), enzymes such as peroxidase and lysozyme (Padgett and others 1998); and polysaccharides with antimicrobial properties like chitosan (Ouattara and others 2000b) have also been tested.

Many polysaccharides have been used for the production of edible packaging; however starch is the most commonly used natural biopolymer. Starch has been found as an interesting alternative for edible films because it is biodegradable, abundant, cheap and easy to use (Mali and others 2002). Starch based films have different properties depending on the amylose content in the starch (Lawton 1996). In 1999, a study was focused on the properties of an edible starch-alginate film where different concentrations of calcium ions were investigated. This study was focused on optical and mechanical properties. Calcium ion concentrations showed a direct relationship with mechanical strength, while elongation of the film showed an indirect relationship. Changes in color, opacity, brightness and yellowness of the film containing calcium

were not found to be significant. However, the presence of clots and tiny air bubbles in the cast films were observed at higher concentration of the calcium ion (Sivanandan and others 1999).

Incorporation of the antimicrobial agents to the film can take several forms. 1) Direct incorporation of the antimicrobial inside the polymer, 2) coating or adsorbing antimicrobials onto polymer surfaces, 3) immobilization of antimicrobials to polymers by ion or covalent linkages, and 4) use of polymers that have antimicrobial properties (Appendini and Hotchkiss 2002). Heat press and casting are specific methods for incorporation of the antimicrobial agents into the films. Other authors studied these two film-forming methods using soy protein and corn zein, with the incorporation of lysozyme and nisin. The films were tested against *Lactobacillus plantarum* (NCDO 1752) and further incorporation of EDTA was evaluated against *E. coli*. Both film-forming methods formed excellent films and the properties of lysozyme and nisin were retained. However cast films showed larger inhibition zones than the heat-press films (Padgett and others 1998).

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CHAPTER 2

THE INHIBITORY EFFECT OF SELECTED LACTIC ACID BACTERIA AND ITS MAIN METABOLIC PRODUCTS ON THE GROWTH OF *LISTERIA MONOCYTOGENES*¹

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Abstract

Lactic acid bacteria (LAB) are known to produce metabolic byproducts having inhibitory activity against spoilage and pathogenic bacteria. The inhibitory effect of lactic acid bacteria cells, lactic acid and bacteriocins on *Listeria monocytogenes* (LM) individually was demonstrated. We hypothesized that bacteriocin is mainly responsible for the initial inhibitory effect when a LAB culture medium containing live cells is added to a LM culture. Eventually LAB cells proliferate and inhibit LM through competitive inhibition. *Listeria* inhibition was achieved with 8 ml of cell-free LAB culture medium supernatant in 10 mL LM culture medium. The live LAB cells had significant impact only after 21 days at 4°C. The MIC to develop an inhibition zone using the agar diffusion assay was 1 ml of cell-free culture medium supernatant for each LAB studied and the main factor responsible for inhibition of *Listeria* was the synergistic effect of lactic acid and bacteriocin. Better inhibition was achieved when pH was modified close to 4.0. *L. carnosum* 4010 produced more active bacteriocins with a lower lactic acid production compared to *L. carnosum* ATCC 49367 and *L. plantarum* ATCC 8014. Results also suggest that the presence of actively growing LAB cells can increase the inhibitory effect against *Listeria*. LAB with potent bacteriocin and low lactic acid production are strong candidates to use as protective cultures to increase safety without changing the quality attributes of ready-to-eat meat products.

Introduction

Lactic acid bacteria (LAB) generally considered as 'food-grade' organisms have shown special promise for selection and implementation as protective cultures (Holzapfel and others 1995). LAB can produce mainly lactic acid and in lower quantities other organic acids, diacetyl, hydrogen peroxide and others (Axelsson 1998); some LAB can also produce bacteriocins (Ouwehand 1998). Ready to eat meat products have been the source for the isolation of some LAB that can inhibit spoilage bacteria and also, if present, pathogens such as *Listeria monocytogenes*. However, the preservation of RTE meat products using LAB can render the product organoleptically unacceptable if the organism produces large amounts of organic acids. Consequently, selection of a suitable LAB and the separation and use of cell-free bacteriocin preparation is a very important part of the process of adopting this biotechnological approach to improve of RTE meat safety.

Many studies have been done to identify the dominant LAB in cooked meat products. The LAB strains generally considered as the natural microflora in meats and meat products are: *Carnobacterium piscicola*, *C. divergens*, *Lactobacillus sakei*, *Lb. curvatus* and *Lb. plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc gelidum* and *Leuconostoc carnosum* (Hugas 1998; Vermeiren and others 2004; Greco and others 2005). *Lactobacillus plantarum* 5 was shown to produce larger inhibition zones compared with 11 other selected strains of LAB identified as *L. sakei* and *L. curvatus* when tested against different *L. monocytogenes* strains (Vermeiren and others 2004). *L. plantarum* ATCC 8014 cell-free supernatant was also tested against : *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Lash and others 2005). Direct application of live cells of *L. carnosum* 4010 on vacuum packed meat sausages showed immediate reduction of *Listeria* to an

undetectable level at 5°C over 21 days. *Leuconostoc carnosum* 4010 was added at a concentration of 10⁷ cfu/g, and *Listeria monocytogenes* was inoculated at ca. 10 cfu/g (Budde and others 2003). Studies on non-bacterinogenic lactic acid bacteria have been also done and different LAB were identified including strains of *L. sakei* (Bredholt and others 1999; Vermeiren and others 2005), *Lactobacillus casei* and *Lactobacillus paracasei* (Amézquita and Brashears 2002).

Listeria monocytogenes can grow at low temperatures, the minimum growth temperature was found to be in a range of 0.5 – 3.0°C (Junttila and others 1988). Due to this ability, refrigeration is inadequate to control *Listeria* growth. In 1990 an outbreak of foodborne illness occurred involving hotdogs and deli (luncheon) meats. As a response of this situation FSIS published a notice advising RTE meat and poultry processors to reassess their HACCP plans to ensure that *L. monocytogenes* problem is adequately addressed (USDA-FSIS 2004). Finally, the U.S. Department of Agriculture Food Safety and Inspection Service set a zero tolerance limit for *L. monocytogenes* in ready to eat foods. The Code of Federal Regulations mentions the use of harmless lactic acid producing bacteria as protective agents in meat products; however no specific microorganisms were named under this regulation (9CFR424.21). To date, nisin is the only bacteriocin that has been accepted by the USDA-FSIS as GRAS (Generally Recognized as Safe) (21CFR184.1538).

LAB show special promise as a protective culture. The ideal LAB should satisfy the following constraints: no health risks, competitiveness against undesired microorganisms, no negative (sensory) effects on product under GMP and function as ‘indicator’ under abuse conditions (Holzapfel and others 1995).

The purposes of this study were to evaluate three LAB strains isolated from RTE meat products in their ability to inhibit *L. monocytogenes*. This study focused on: first the effectiveness of LAB cells and the cell-free supernatant separately and in combination; second the Minimum Inhibitory Concentration to produce a positive zone of inhibition using the agar diffusion technique and third the determination of the main components responsible for *Listeria* inhibition. These studies resulted in the identification of a LAB with superior characteristics as a protective culture in RTE meats.

Materials and Methods

Bacterial strains and culture conditions:

The LAB used were *Lactobacillus plantarum* ATCC 8014, *Leuconostoc carnosum* ATCC 49367 and *Leuconostoc carnosum* 4010 (B-SF-43 Bactoferm™, CHR HANSEN). *Listeria monocytogenes* Scott A, obtained from the Center of Food Safety, The University of Georgia, Griffin, GA, was the only listerial strain used during this experiment. The stock cultures were kept on Microbank™ beads (Pro-Lab diagnostics, Austin, Tex., USA) at -80°C.

All LAB were activated in 9 ml of DeMan-Rogosa-Sharpe broth (MRS, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.). *L. plantarum* was incubated at 37°C while *L. carnosum* strains were incubated at 25°C, for 18 to 24 h. *L. monocytogenes* was activated using 9 ml of tryptic soy broth (TSB, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.) at 37°C for 18 to 24 h. There were three consecutive transfers of the cultures before use.

The inhibitory effects of LAB's cell-free supernatant and live LAB cells against *Listeria monocytogenes*

The activity of LAB (*L. plantarum* ATCC 8014, *L. carnosum* ATCC 49367 and *L. carnosum* 4010) against *L. monocytogenes* Scott A was tested at 4°C in broth under anaerobic conditions (5% O₂, 10% CO₂ and 85% N) for four weeks.

Each active growing culture (9 ml) was centrifuged (BeckMan Coulter™ Allegra™ X-22R Centrifuge, Palo Alto, California, U.S.A) at 7000 x g for 10 min at 4°C; the supernatant was decanted and saved and the pellet was re-suspended in 0.1% peptone water. Cell suspensions in 0.1% peptone were prepared to contain the desired concentrations. The LAB cell free supernatant was obtained after further filtration using a sterile filter with a pore size of 0.45 µm.

Four treatment groups with different concentrations of LAB cells were used: 10⁵, 10⁶, 10⁷ and 10⁸ cfu/ml for *L. plantarum* ATCC 8014 and *L. carnosum* 4010; and 10⁴, 10⁵, 10⁶ and 10⁷ cfu/ml for *L. carnosum* ATCC 49367. *L. monocytogenes* was evaluated in three levels: 10¹, 10², and 10³ cfu/ml.

Each level of *L. monocytogenes* had two controls: control 0 contained 9 ml of MRS broth and 1 ml of peptone with *L. monocytogenes* at the designated level; control 1, contained 8 ml of supernatant from the respective Lactobacillus culture studied + 1 ml of peptone containing *Listeria* cells at a specific level + 1 ml of 0.1% peptone to complete the 10 ml volume.

Treatments and the designated symbols are as follows: Treatments containing cells at levels of 10⁵, 10⁶, 10⁷ and 10⁸ cfu/ml for *L. plantarum* were designated LP-5, LP-6, LP-7 and LP-8; for *L. carnosum* 4010 were designated LC-5, LC-6, LC-7 and LC-8; finally, treatments with *L. carnosum* ATCC at levels of 10⁴, 10⁵, 10⁶ and 10⁷ cfu/ml were designated Lc-4, Lc-5, Lc-6 and Lc-7. Each treatment group contained: 8ml of cell-free supernatant + 1 ml of MRS

LAB (*L. plantarum*, *L. carnosum* 4010 or *L. carnosum* ATCC) at the specified cell concentration + 1 ml of 0.1% peptone water with *L. monocytogenes* at the specified concentration 10^1 , 10^2 , and 10^3 cfu/ml, designated as follow: L-1, L-2, and L-3.

Control 0 showed the change in numbers of *L. monocytogenes* in MRS broth at 4°C. Control 1 showed the effect of cell-free supernatant against *L. monocytogenes*. Finally, the treatments were used to see the inhibitory effect of different concentrations of live LAB cells against *Listeria*. Inoculation levels of 10 and 100 cfu/ml of *L. monocytogenes* were used and the medium was analyzed for presence or absence of *L. monocytogenes*. Aerobic CFU ≥ 1000 cfu/ml was determined using tryptic soy agar. Presence of viable *Listeria* cells (positive or negative) was evaluated using the USDA's enrichment procedure using *Listeria* enrichment broth (UVM, Difco™, Division of Becton Dickinson and co., Sparks, Md., U.S.A.), Fraser broth base (Fraser Broth, Difco™, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.), and Modified oxoid agar (MOX; Oxoid; Basingstoke, Hampshire, England) plates. Presumptive positive *Listeria* colonies were further identified using the catalase and motility tests. Samples were taken after 7, 14, 21, and 28 days. Three replications per LAB strain studied were made.

Minimum Inhibitory Concentration (MIC)

Different volumes of cell-free supernatant were tested against *L. monocytogenes*. After centrifugation the supernatant was used in the agar diffusion test. Sterilized 18 mm dia. glass wells were used. The MIC was defined as the minimum volume of supernatant able to produce a positive inhibition zone. The inhibition zone was considered positive if the distance of the clear zone was 0.5 mm or more measured from the exterior border of the well (Fleming and others 1975). An active culture of *L. monocytogenes* was serially diluted to 10^4 CFU/ml and pour plated into TSA at 50°C. After the agar solidified, four sterilized glass wells were placed in the agar.

Enough space was left between wells to avoid overlap of the zones of inhibition around each well. Finally, 0.5, 0.8 and 1.0 ml of the supernatant was added into individual wells. One control well contained 1 ml of 0.5% peptone water. The three LAB strains were studied. The experiment was replicated three times.

The following tests: Inhibition of hydrogen peroxide, lactic acid and enzyme test were done following the methodology used by Lewus and others (1991).

Inhibition of hydrogen peroxide production in LAB culture broth

Inhibition due to hydrogen peroxide was avoided by growing each lactic acid bacteria under anaerobic conditions created using a gas with the following composition: 5%O₂, 10%CO₂ and 85% N. Agar diffusion test was used. Overnight cultures (18-24 h) of *L. monocytogenes* and LAB were used for the experiment. *Listeria* was poured plated into TSA at a concentration of 10⁴cfu/ml. After the agar solidified, sterile glass wells were placed on the agar (4 wells per plate). One ml of each lactic acid bacteria stock culture was inoculated inside the wells and one well was filled with peptone water as a control. The plates were incubated in anaerobic conditions at 37°C for 24 h. After incubation, the plates were examined for the presence of inhibition zones around the wells. Three replications were made per each LAB studied. This same experiment was repeated under aerobic conditions.

Inhibition of lactic acid production by LAB

An actively growing LAB stock culture was used to inoculate test tubes containing 9 ml of TSBYE (tryptic soy broth without dextrose with 0.5% yeast extract supplement; Difco™). At the same time tubes containing MRS broth were inoculated in order to be able to compare the effect of lactic acid on the inhibitory properties against *Listeria* of the LAB culture medium. Each LAB strain was incubated at 25°C and 37°C for *L. carnosum* and *L. plantarum*, respectively

for 24 h, then the test tubes were centrifuged at 7000 x g for 10 min at 4°C and the cell-free supernatant was decanted and saved.

An active culture of *L. monocytogenes* was consecutively diluted until 10^4 cfu/ml was reached , and then 1 ml was pour plated into TSA at 45-50°C. The agar diffusion test was used and wells were placed inside each Petri plate after the TSA was solidified. The number of wells used per plate was the same previously described.

a) Effect of the supernatant

Three wells were used per plate. Well #1 was inoculated with 1 ml of TSBYE cell-free supernatant, well #2 was inoculated with 1 ml of MRS cell-free supernatant and finally the well #3 was inoculated with 1 ml of 0.1% peptone water as a control. The plates were incubated at 37°C for 24 h in aerobic conditions and zones of inhibition were examined. Three replications were made for each LAB strain analyzed.

b) Effect of lactic acid cells

The effect of live lactic acid cells was tested. This test was made in order to compare the effect of lactic acid cells and supernatant together, and the effect of the cell-free supernatant in a medium where no lactic acid is present (TSB without Dextrose). Three wells were placed in the Petri plate used in the agar diffusion test. One was filled with 1 ml of the TSBYE broth from a 24 h culture of lactic acid bacteria (either 10^8 cfu/ml for *L. carnosum* ATCC 49367 or 10^9 cfu/ml for each *L. carnosum* 4010 and for *L. plantarum* ATCC 8014). A second well contained 1 ml of the cell-free supernatant obtained after centrifugation of the TSBYE broth. The control well contained 1 ml of 0.1% peptone water.

c) pH effect

The effect of pH on bacteriocin activity was tested. TSBYE was adjusted to a pH of approximately 4 before inoculation of the LAB. The pH adjustment was achieved using 1N HCl, where the original pH value of the media was approximately 7. A pH meter (Fisher Scientific™ Accumet AB15, Pittsburgh, PA) was used. After pH modification, the tubes were inoculated with lactic acid bacteria and then incubated at 25°C for both strains of *L. carnosum* and 37°C for *L. plantarum* for 24 h. Cell-free supernatant was obtained by centrifugation of these overnight cultures at 7000 x g for 10 min at 4°C. The supernatant was then filter using a 0.45 µm sterilized syringe filters. The pH of the collected supernatant was measured. The agar diffusion test was used to compare the effect of pH on the activity of the bacteriocin. Three wells were used per plate. One well was filled with 1 ml of the collected cell-free supernatant, the second contained supernatant with cells and the third one was the control with 1 ml of 0.1% peptone water. Different petri plates were used for each LAB strain. Three replications were made per LAB strain studied. A similar experiment was done at the same time with a supernatant which pH was not modified. The final pH of the TSBYE after inoculation and incubation was measured.

Enzyme test: Sensitivity of bacteriocin to proteolytic enzyme

The protein nature of the bacteriocin was assessed by determining the loss of activity after treatment with a proteolytic enzyme. Active MRS growing cultures (18-24 h) of LAB was used to inoculate test tubes containing 9 ml of TSBYE. These tubes were incubated at 25 and 37°C for 24 h for *L. carnosum* strains and *L. plantarum*, respectively. Cell-free supernatant was obtained after centrifugation and filtration as previously described. Serial dilutions of an active *Listeria* culture were made until 10⁴cfu/ml was achieved, then 1 ml of this broth was pour plated into TSA at 45-50°C. The proteases used were: Proteinase k (fungal type XI), Protease

(*Streptomyces griseus* type XIV), and α -chymotrypsin (bovine pancreas, type II) (all from Sigma Chemical Co., St. Louis, Mo.). The enzymes were prepared in the designated concentrations (10 mg/ml and 1 mg/ml) a day prior to the experiment. Experiments were carried out with an enzymatic reaction time of 1, 3, 12, and 24 h. To 0.9 ml of each LAB cell free supernatant, 0.1 ml of Proteinase K (10 mg/ml), Protease (10 mg/ml) and α -chymotrypsin (10 mg/ml) was added in different vials; the supernatant was then incubated for 1, 3, 12, and 24 h at 30°C. One ml of the digested cell-free supernatant was inoculated inside the wells. Four wells were used per Petri plate. Three replications were made per each LAB strain studied.

Inhibition zone produced by bacteriocin

The preceding experiment was repeated using MRS broth cultures of LAB in order to see zones of *Listeria* inhibition by lactic acid. The inhibition zone produced by the enzyme treated bacteriocins was obtained indirectly as the difference between width of inhibition zones induced by MRS culture broth supernatant minus that produced by the enzyme treated supernatant. Four wells were used per plate, three contained the supernatant digested by each of the enzymes and a fourth one (control) contained the untreated supernatant.

Statistical Analysis

The average plate counts, obtained for *L. carnosum* ATCC 49367 at the higher level of *Listeria* inoculation (10^3 CFU/ml), were transformed to logarithmic counts and analyzed for significant differences ($p < 0.05$). The analysis were conducted using PROC GLM procedure in the Statistical Analysis System (SAS, 2001). A randomized complete block design was performed.

The average values of the width of inhibition zones due to bacteriocin and the effect of each enzyme were analyzed using a complete random design.

Results and Discussions

All three LAB tested showed different degree of inhibitory activity against *L. monocytogenes* Scott A. *L. carnosum* ATCC 49367 reduced *Listeria* population to below the detection level (1 CFU/ml) after 28 days incubation at 4°C (Table 2.1). The inhibitory action was effective on three levels of *Listeria* inoculation 10^1 , 10^2 and 10^3 CFU/ml. *L. carnosum* ATCC 49367 cell-free supernatant appeared to be just as effective as the culture broth containing the cells. This effect was seen during the first 2 weeks of incubation. There was no significant difference ($p > 0.05$) in effects of the cell-free culture broth supernatant and those of the culture broth with cells. Cell-free culture broth supernatant and the culture broth with cells at all levels of LAB cells added seem to be equally effective during the first 2 weeks of incubation. However, *Listeria* inhibition was significantly different ($p < 0.05$) after 21 days when the level of LAB cells was 10^7 CFU/tube. The reason for this could be the slow growth rate of the LAB cells at 4°C, since no significant LAB growth was achieved during 7 and 14 days. However after 21 days enough metabolites were expressed into the medium by the larger cell numbers increasing the inhibitory effect against *Listeria*. *L. plantarum* ATCC 8014 and *L. carnosum* 4010 achieved complete inhibition of *Listeria* after 7 days of incubation at 4°C, as indicated by the negative presence of *Listeria* after USDA's enrichment procedure. The inhibitory activity was maintained over the 4 weeks of the study at the three levels of *Listeria* tested (Table 2.2 and 2.3).

Minimum Inhibitory Concentration (MIC)

In the well diffusion assay, 1ml of cell-free supernatants was able to produce a clear positive inhibition zone. *L. plantarum* ATCC 8014 produced the largest inhibition zone, followed by *Lecuoconostoc carnosum* 4010 and *L. carnosum* ATCC 49367. A *L. plantarum* strain (LP5) has previously been demonstrated to have stronger inhibitory activity against *L.*

monocytogenes Scott A compared with other 11 LAB strains identified as *L. sakei* and *L. curvatus* (Vermeiren and others 2004). Less volume of supernatant (0.5 and 0.8 ml) did not form zones of inhibition; however some clearing can be observed inside the well (not shown). The results suggested that 1 ml of supernatant was the minimum inhibitory concentration (MIC) for the three LAB strains tested against *L. monocytogenes*.

Effect of hydrogen peroxide production on inhibitory activity of LAB cultures

The zones of inhibition obtained under anaerobic conditions were similar results to those under aerobic conditions (not shown). The cell-free supernatant from *L. plantarum* ATCC 49367 produced the larger inhibition zones, followed by *L. carnosum* 4010 and *L. carnosum* ATCC 8014. These results show that hydrogen peroxide production by the LAB was not responsible for *Listeria* inhibition.

Effect of Lactic Acid production on inhibitory activity of LAB cultures

Lactic acid production by the LAB was prevented by growing the organisms in TSBYE broth. No inhibition zones were detected outside the wells on any of the LAB cultured in TSBYE broth. Some clearing was visible inside the wells.

Inhibitory activity of live LAB cells

The wells containing LAB cells showed more clearing of *Listeria* colonies inside the well compare with those with only supernatant inside the wells (Figure 2.5, 2.6 and 2.7). The reason could be the possible competition for nutrients between LAB and *Listeria* or due to the additional production of bacteriocin by the live cells during the incubation period (24 h). The three LAB tested showed the same effect.

Effect of pH on inhibitory activity

After pH adjustment of the TSBYE broth, the supernatant of a 24 h culture showed better antilisterial activity. The final pHs of the collected supernatant after pH adjustment were: 4.92, 4.84, and 4.90 for *L. plantarum*, *L. carnosum* 4010 and *L. carnosum* ATCC 49367 respectively. At the same time the final pH of the TSBYE cell-free supernatant without pH adjustment were 6.12, 6.04 and 6.13 for *L. plantarum*, *L. carnosum* 4010 and *L. carnosum* ATCC 8014 respectively. The bacteriocins seemed to be more active when the pH is adjusted to be close to pH 4.0 (Figure 2.6 and 2.7). The density of *Listeria* survivors at pH around 7 (Figure 2.5, Well #1) was higher than the one at pH 4 (Figure 2.6, Well #1). These results confirmed that bacteriocin activity was better with a pH around 4 than one around 7. These results agreed with other previous studies where *L. plantarum* was shown to have better bacteriocin activity at a pH between 4 and 5 (Lash and others 2005). The effect of pH on *Listeria* inhibition was similar in all three LAB under study. However, the intensity of the inhibition among the three LAB varied. TSBYE cell-free supernatant from *L. carnosum* 4010 showed the strongest *Listeria* inhibition at reduced pH (See Figure 2.7).

Effect of proteolytic enzymes on inhibitory activity of LAB culture broth against *Listeria*

Substances produced by the LAB which possessed inhibitory activity against *Listeria* lost their activity when the LAB culture broths were treated with the proteolytic enzymes for 24 h. The culture broth supernatants enzyme treated for 24 h contained a white precipitate in each vial. Treatment times of 1, 3 and 12 h did not develop the white precipitate. The treated cell-free supernatants were used in the well diffusion assay, and they showed no inhibition zones. There was growth of *Listeria* inside the wells (Figure 2.8). The responses of the LAB culture

supernatants when treated with the enzyme confirmed the proteinaceous nature of the bacteriocins produced by the three LAB strains studied.

Zones of Inhibition on agar diffusion wells due to bacteriocins

The width of the inhibition zones around agar diffusion wells containing the bacteriocins from *L. carnosum* 4010, *L. carnosum* ATCC 49367 and *L. plantarum* ATCC 8014 can be seen in Figures 2.9, 2.10 and 2.11 respectively. Table 2.4 shows an example of how the value of the width of inhibition zone due to bacteriocin was obtained. The average values of the width of the Inhibition zone due to bacteriocin seem to indicate that *L. carnosum* 4010 produced the most effective bacteriocins (Table 2.5), however the statistical analysis showed no significant difference ($p > 0.05$) between inhibition zones of all bacteriocins studied. There was also no difference in the action of the different proteolytic enzymes on the bacteriocins.

Conclusions

All three lactic acid bacteria studied produced metabolic products inhibitory against *L. monocytogenes* Scott A. The most effective LAB were *L. plantarum* ATCC 8014 and *L. carnosum* 4010 which reduced the population of *Listeria* to undetectable levels after 7 days of treatment at 4°C. Under the conditions of the experiment, the presence of live LAB cells was not necessary for *Listeria* inhibition if adequate cell-free supernatant was present. If not enough of the cell-free supernatant is added to the *Listeria* culture broth, the LAB cells (10^7 CFU/ml) will have significant impact on *Listeria* inhibition only after 21 days. The ratio of cell-free supernatant to *Listeria* broth culture 10^3 CFU/ml was 8:1 v/v. In the agar diffusion test, the minimum inhibitory concentration of culture broth from all three LAB strains studied to produce inhibition zones around the wells was 1 ml, when *Listeria* was present at ca. 100 CFU/cm² and

the plates incubated at 30°C for 24 h. *Listeria* was inhibited by two of the LAB metabolites, the bacteriocin and lactic acid. The best inhibitory activity resulted when metabolites were added to the *Listeria* culture. In the absence of lactic acid and only bacteriocin from the LAB culture was the main inhibitory compound, the presence of live cells was necessary to obtain maximum *Listeria* inhibition. Bacteriocins activity is maximum at pH 4 to 5. Overall, *L. carnosum* 4010 seems to be more effective than the other LAB bacteria studied for *Listeria* inhibition in foods because its bacteriocin is more active and the low lactic acid production minimizes off-flavor development.

Table 2.1 Effect of *L. carnosum* ATCC 49367 against *L. monocytogenes* Scott A (Level: 10¹, 10² and 10³ CFU/ml).

| <i>Listeria</i> (log ₁₀ CFU/ml) | <i>L. carnosum</i> ATCC 49367 | | | | |
|---|-------------------------------|--------------|---------------|---------------|--------------|
| | Day 7 | | | | |
| | Control 1 | Lc-4 | Lc-5 | Lc-6 | Lc-7 |
| 1.58 ± 0.42 ^a | 3/3 ^d | 3/3 | 3/3 | 3/3 | 3/3 |
| 2.51 ± 0.14 ^b | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 3.52 ± 0.29 ^c | 2.80 ± 0.16 ^c | 2.82 ± 0.08 | 2.80 ± 0.15 | 3.00 ± 0.20 | 2.80 ± 0.10 |
| | Day 14 | | | | |
| | Control 1 | Lc-4 | Lc-5 | Lc-6 | Lc-7 |
| | | | | | |
| 1.25 ± 0.23 | 1/3 | 2/3 | 3/3 | 3/3 | 3/3 |
| 2.20 ± 0.17 | 3/3 | 3/3 | 2/3 | 2/3 | 1/3 |
| 3.26 ± 0.13 | 2.31 ± 0.22 | 2.33 ± 0.15 | 2.21 ± 0.24 | 2.26 ± 0.36 | 2.35 ± 0.34 |
| | Day 21 | | | | |
| | Control 1 | Lc-4 | Lc-5 | Lc-6 | Lc-7 |
| | | | | | |
| 1.23 ± 0.48 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 |
| 2.25 ± 0.18 | 3/3 | 2/3 | 2/3 | 2/3 | 3/3 |
| 3.25 ± 0.11 | 1.42 ± 0.21fg | 1.79 ± 0.16f | 1.50 ± 0.38fg | 1.48 ± 0.26fg | 1.35 ± 0.12g |
| | Day 28 | | | | |
| | Control 1 | Lc-4 | Lc-5 | Lc-6 | Lc-7 |
| | | | | | |
| 1.40 ± 0.45 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.23 ± 0.15 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.28 ± 0.06 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |

^a *Listeria* inoculated: ca. 10 CFU/ml

^b *Listeria* inoculated: ca. 100 CFU/ml.

^c *Listeria* inoculated: ca. 1000 CFU/ml.

^d Positive tubes over the number of tubes tested after USDA's *Listeria* enrichment procedures.

^e Results from cell count procedure (log₁₀ CFU/ml)

f,g: different letters on the same row are significantly different (p < 0.05).

Table 2.2 Effect of *L. plantarum* ATCC 8014 against *L. monocytogenes* Scott A (Level: 10^1 , 10^2 and 10^3 CFU/ml).

| <i>Listeria</i> (log ₁₀ CFU/ml) | <i>L. plantarum</i> ATCC 8014 | | | | |
|---|-------------------------------|------|------|------|------|
| | Day 7 | | | | |
| | Control 1 | LP-5 | LP-6 | LP-7 | LP-8 |
| 1.46 ± 0.21^a | 0/3 ^d | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.38 ± 0.16^b | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.46 ± 0.11^c | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Day 14 | | | | | |
| | Control 1 | LP-5 | LP-6 | LP-7 | LP-8 |
| 1.53 ± 0.34 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.32 ± 0.11 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.35 ± 0.18 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Day 21 | | | | | |
| | Control 1 | LP-5 | LP-6 | LP-7 | LP-8 |
| 1.46 ± 0.25 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.42 ± 0.12 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.32 ± 0.13 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Day 28 | | | | | |
| | Control 1 | LP-5 | LP-6 | LP-7 | LP-8 |
| 1.35 ± 0.13 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.37 ± 0.16 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.31 ± 0.05 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |

^a *Listeria* inoculated: ca. 10 CFU/ml

^b *Listeria* inoculated: ca. 100 CFU/ml.

^c *Listeria* inoculated: ca. 1000 CFU/ml.

^d Positive tubes over the number of tubes tested after USDA's *Listeria* enrichment procedures.

Table 2.3 Effect of *L. carnosum* 4010 against *L. monocytogenes* Scott A (Level: 10¹, 10² and 10³ CFU/ml).

| <i>Listeria</i> (log ₁₀ CFU/ml) | <i>L. carnosum</i> 4010 | | | | |
|---|-------------------------|------|------|------|------|
| | Day 7 | | | | |
| | Control 1 | LC-5 | LC-6 | LC-7 | LC-8 |
| 1.39 ± 0.21 ^a | 0/3 ^d | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.45 ± 0.09 ^b | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.41 ± 0.21 ^c | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| | Day 14 | | | | |
| | Control 1 | LC-5 | LC-6 | LC-7 | LC-8 |
| | | | | | |
| 1.45 ± 0.13 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.35 ± 0.11 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.34 ± 0.17 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| | Day 21 | | | | |
| | Control 1 | LC-5 | LC-6 | LC-7 | LC-8 |
| | | | | | |
| 1.61 ± 0.15 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.31 ± 0.17 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.31 ± 0.16 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| | Day 28 | | | | |
| | Control 1 | LC-5 | LC-6 | LC-7 | LC-8 |
| | | | | | |
| 1.31 ± 0.24 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2.24 ± 0.16 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3.29 ± 0.11 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |

^a *Listeria* inoculated: ca. 10 CFU/ml

^b *Listeria* inoculated: ca. 100 CFU/ml.

^c *Listeria* inoculated: ca. 1000 CFU/ml.

^d Positive tubes over the number of tubes tested after USDA's *Listeria* enrichment procedures.

Table 2.4 Indirect measurement of *Listeria* inhibition by bacteriocin from *L. carnosum* 4010.

| Well # | Enzyme used | Inhibition zone (from the border of the well) | Inhibition zone due to Bacteriocin |
|--------|-----------------------------|---|------------------------------------|
| 1 | Protease | 0.5 mm | $3.5 - 0.5 = 3.0$ mm |
| 2 | Proteinase- k | 1.0 mm | $3.5 - 1.0 = 2.5$ mm |
| 3 | α -chymotrypsin | 1.0 mm | $3.5 - 1.0 = 2.5$ mm |
| 4 | Control (supernatant alone) | 3.5 mm | |

Table 2.5 Inhibitory effect of bacteriocin from *L. plantarum* ATCC 8014, *L. carnosum* ATCC 49367 and *L. carnosum* 4010.

| Enzyme | Zones of Inhibition (mm) | | |
|------------------------|----------------------------------|----------------------------------|----------------------------|
| | <i>L. plantarum</i> ATCC 8014 | <i>L. carnosum</i> ATCC 49367 | <i>L. carnosum</i> 4010 |
| Protease | 2.0 | 1.7 | 2.5 |
| Proteinase k | 2.0 | 2.0 | 2.7 |
| α -chymotrypsin | 1.8 | 1.7 | 2.5 |
| Overall average | 1.9 ± 0.1 | 1.8 ± 0.2 | 2.6 ± 0.1 |

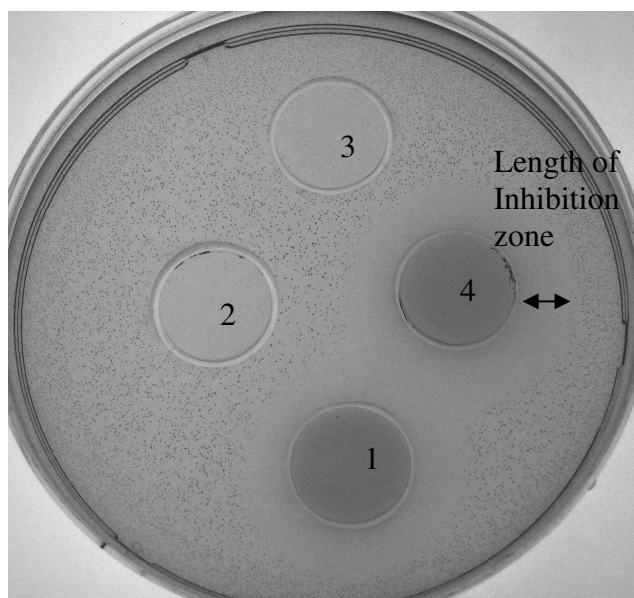


Figure 2.1 Inhibition zones: *L. carnosum* 4010 and *L. carnosum* ATCC 49367 in MRS and TSBYE broth.

- *Well #1 *L. carnosum* ATCC 49367 cell-free supernatant (MRS broth)
- *Well #2 *L. carnosum* 4010 cell-free supernatant (TSBYE broth)
- *Well #3 *L. carnosum* ATCC 49367 cell-free supernatant (TSBYE broth)
- *Well #4 *L. carnosum* 4010 cell-free supernatant (MRS broth)

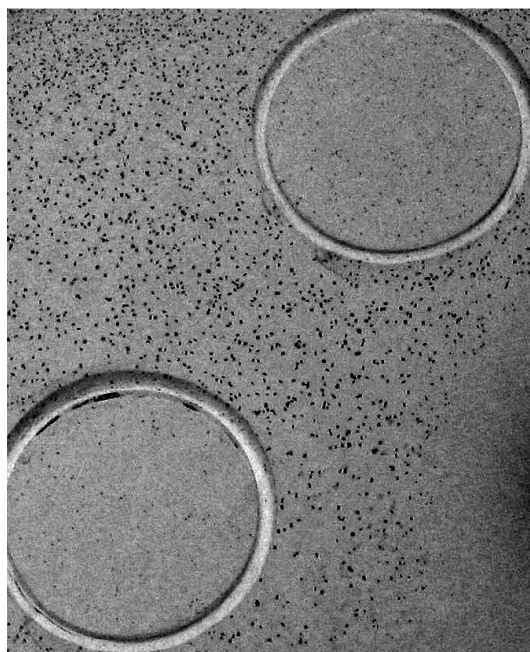


Figure 2.2 Effect of bacteriocin from *L. carnosum* 4010 and ATCC 49367 (Zoom of Well #2 and #3 from Figure 2.1).

- *Well #2 bottom (*L. carnosum* 4010 in TSBYE)
- *Well #3 upper (*L. carnosum* ATCC 49367 in TSBYE)

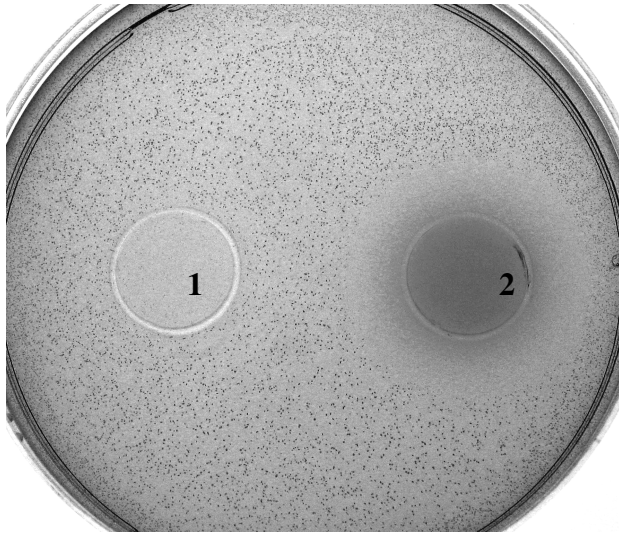


Figure 2.3 Inhibition zones: *L. plantarum* ATCC 8014 in MRS and TSBYE broth.

- Well #1: TSBYE cell-free supernatant
- Well #2: MRS cell-free supernatant

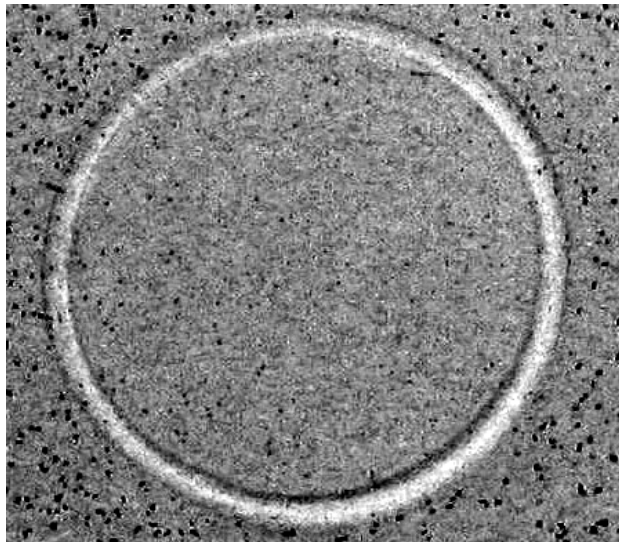


Figure 2.4 *L. plantarum* ATCC 8014 (Zoom of well 1 from picture 2.3).

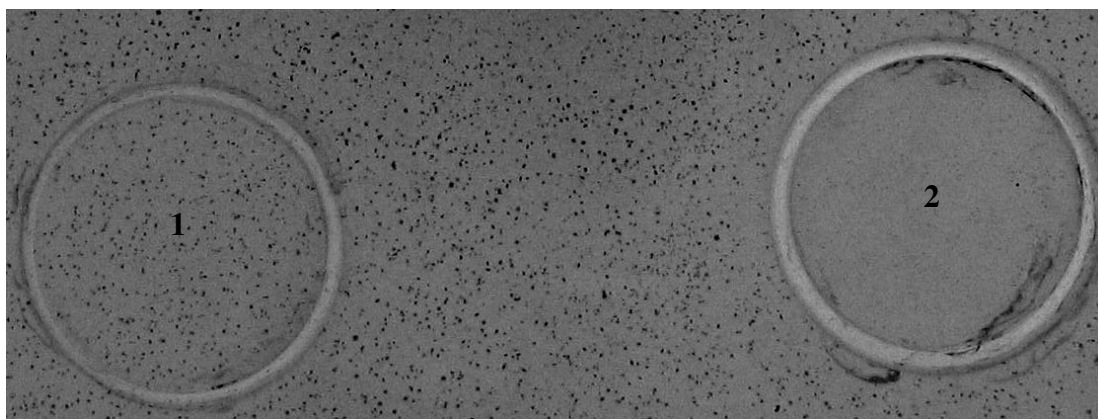


Figure 2.5 Effect of viable LAB cells (*L. plantarum* ATCC 8014) against *L. monocytogenes*.

*Well #1: Cell-free supernatant (TSBYE, pH \approx 7)

*Well #2: Supernatant + cells (10^9 CFU/ml)

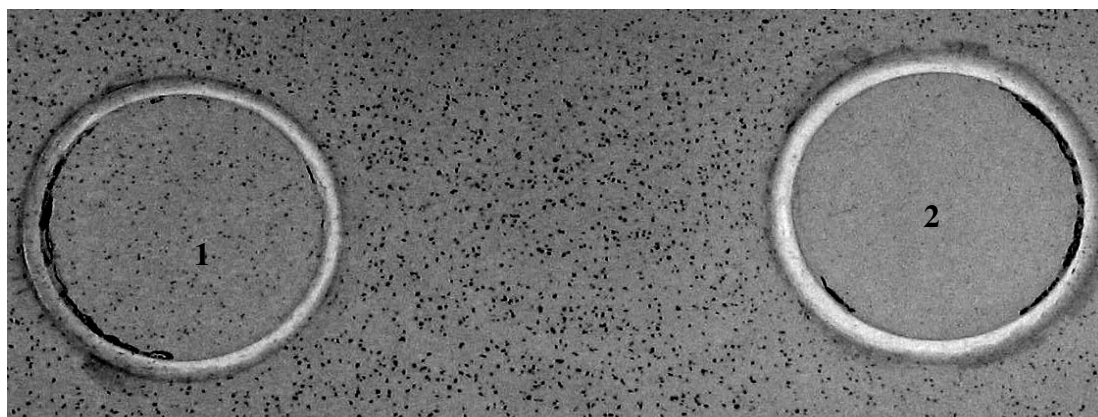


Figure 2.6 Effect of viable LAB cells (*L. plantarum* ATCC 8014) and pH modification against *L. monocytogenes*.

*Well #1: Cell-free supernatant (TSBYE, pH \approx 4)

*Well #2: Supernatant + cells (10^9 CFU/ml)

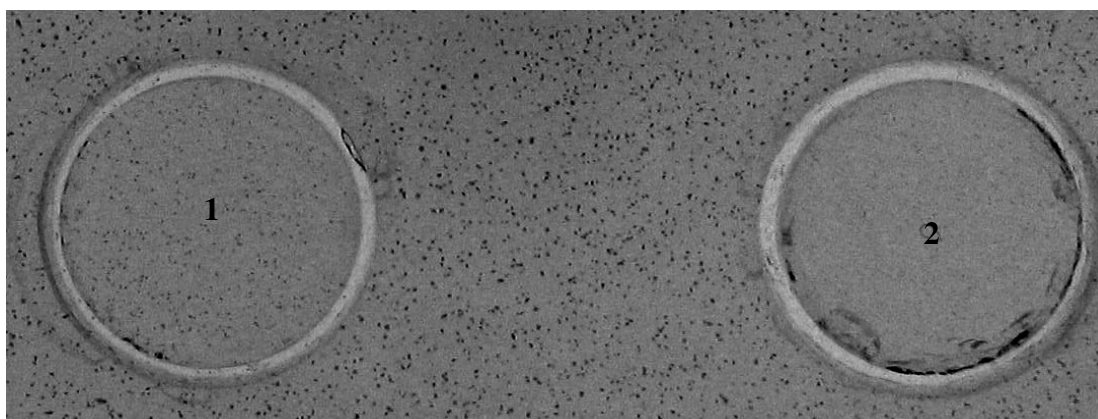


Figure 2.7 Effect of viable LAB cells (*L. carnosum* 4010) and pH modification against *L. monocytogenes*.

*Well #1: Cell-free supernatant (TSBYE, pH \approx 4)

*Well #2: Supernatant + cells (10^9 CFU/ml)

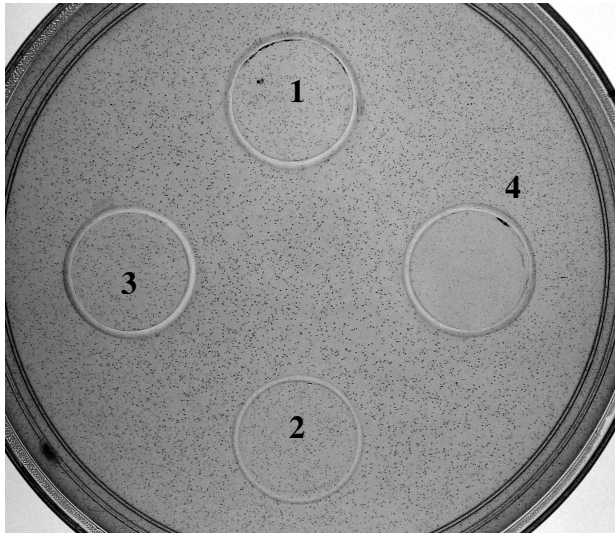


Figure 2.8 Protein nature of bacteriocin (*L. carnosum* 4010) – TSBYE broth – pH \approx 7

Well #1 = cell-free supernatant TSBYE, digested overnight with Proteinase K (10 mg/ml) → No inhibition
 Well #2 = cell-free supernatant TSBYE, digested overnight with α -chymotrypsine (10 mg/ml) → No inhibition
 Well #3 = cell-free supernatant TSBYE, digested overnight with Protease (10 mg/ml) → No inhibition
 Well #4 = TSBYE cell-free supernatant + cells (10^9 CFU/ml) → Significant inhibition

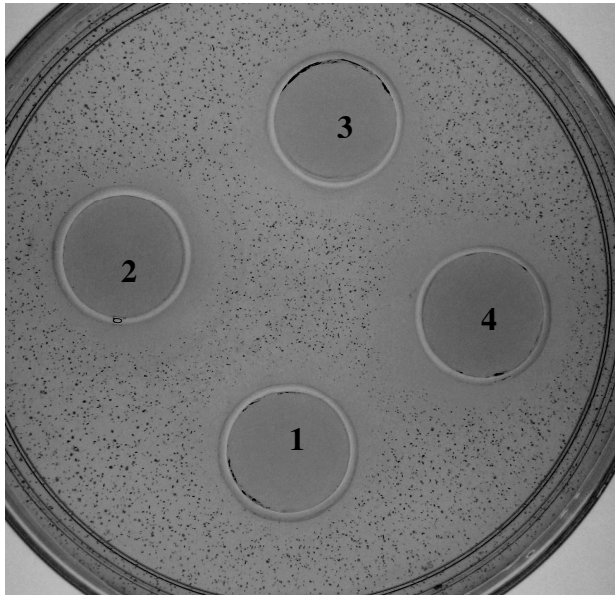


Figure 2.9 Inhibition zones produce by bacteriocin from *L. carnosum* 4010.

*Well #1: MRS cell-free supernatant + Protease
 *Well #2: MRS cell-free supernatant + Proteinase-K
 *Well #3: MRS cell-free supernatant + α -chymotrypsin
 *Well #4: MRS cell free supernatant

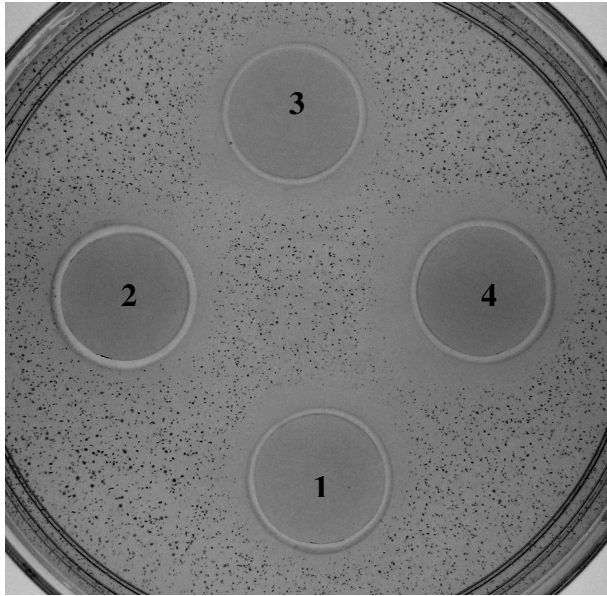


Figure 2.10 Inhibition zones produced by bacteriocin from *L. carnosum* ATCC 49367.

- *Well #1: MRS cell-free supernatant + Protease
- *Well #2: MRS cell-free supernatant + Proteinase-K
- *Well #3: MRS cell-free supernatant + α -chymotrypsin
- *Well #4: MRS cell-free supernatant

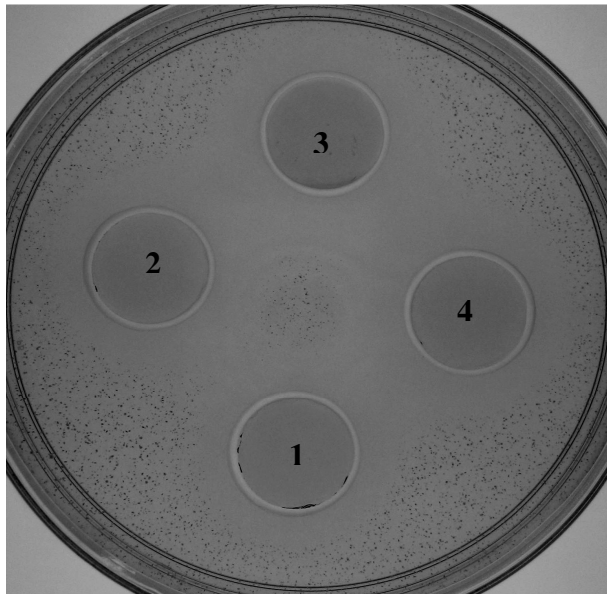


Figure 2.11 Inhibition zone produced by bacteriocin from *L. plantarum* ATCC 8014.

- *Well #1: MRS cell-free supernatant + Protease
- *Well #2: MRS cell-free supernatant + Proteinase-K
- *Well #3: MRS cell-free supernatant + α -chymotrypsin
- *Well #4: MRS cell-free supernatant

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CHAPTER 3

CONTROL OF *LISTERIA MONOCYTOGENES* IN READY TO EAT MEAT PRODUCTS USING ANTIBACTERIAL FILM¹

¹Mora-Cossio, D.; R.T. Toledo and M.A. Harrison. To be submitted to J. Food Protection.

Abstract

Antibacterial films were developed and applied on turkey rolls, a ready-to-eat (RTE) meat product, to test their ability to inhibit growth of *L. monocytogenes* Scott A. *L. carnosum* 4010 (cells and metabolic products) was selected based on previous work as the antimicrobial component to be incorporated into the film. Four methods of antimicrobial incorporation into the film were tested, the best was selected and the films were made in three different thickness. Agar diffusion assay was used to test the antimicrobial starch based films. The surface of turkey rolls were inoculated with three different levels of *L. monocytogenes* Scott A (10^2 , 10^3 and 10^5 CFU/0.1 ml), placed in contact with the selected films and stored for 14 days at 4°C. Modified Oxford agar (MOX) was used for *Listeria* CFU enumeration. The presence of viable *Listeria* was verified using USDA's enrichment procedure for isolation of *L. monocytogenes* and Micro ID™ system for *Listeria* identification. Antimicrobial powder was added before film casting and sterile tape maintained integrity of the film. Two thickness of films: B (0.21 ± 0.03 mm) and C (0.51 ± 0.02 mm) were selected due to the formation of the largest inhibition zones in agar diffusion assay. After the 14 days of storage, *L. monocytogenes* tested positive for all the inoculation levels of *Listeria* regardless of the thickness of film used. However, Film C produced the highest degree of inhibition after 14 days of treatment; almost $2 \log_{10}$ CFU/25 cm² and $1.5 \log_{10}$ CFU/25 cm² reduction of *Listeria* when the initial inoculation was 10^3 CFU/25 cm² and 10^5 CFU/25 cm², respectively.

Introduction

The decrease demand for formulated foods with chemical preservatives and the increasingly requirement for more “natural” and “minimally processed” foods (Cleveland and others 2001), are reasons that have intensified the research on antimicrobial packaging technologies (Suppakul and others 2003). In June of 2003, another reason was added when the FDA/FSIS incorporated in the Code of Federal Regulations the Final Rule 68 FR 34224 entitled as: “Control of *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat Products” (9CFR430.4). This rule mandates that meat and poultry processors must follow one of the three alternatives proposed in order to control *L. monocytogenes* in RTE products. The first alternative calls for the application of both post-lethality treatment and a growth inhibitor for *Listeria* on RTE products, the second alternative calls for the use of one of them, and the third one calls for the application of sanitation measures only. Consequently, antimicrobial packaging provides a choice for the food industry whereby natural antimicrobial agents can be used to ensure the safety of foods without changing their preferred attributes of flavor and texture.

Antimicrobial packaging is a promising form of active food packing, especially for RTE meat products, since post-process contamination occurs mainly at the surface. Antimicrobial agents may be incorporated in the film using either of the following techniques: 1) direct incorporation of the antimicrobial inside the polymer, 2) coating or adsorbing antimicrobials onto polymer surfaces, 3) immobilization of antimicrobials to polymers by ion or covalent linkages, and 4) use of polymers that have antimicrobial properties (Appendini and Hotchkiss 2002).

Packaging films containing antimicrobials have proven to be more effective than other methods for pathogen control in ready to eat products. When the antimicrobial is on a film that

directly contacts the surface of the meat its concentration is maximum on the meat surface while diffusion is slow from the surface towards the interior of the meat (Ouattara and others 2000a). Some studies have also shown that the application of antibacterial agents into meat formulations may result in partial inactivation of the active substances due to interactions with the meat constituents and also due to processing effects like denaturation of the bacteriocins by high temperatures; consequently only a limited amount of antimicrobial agents are available to act on the surface flora (Ouattara and others 2000b). Application of antimicrobial agents only on the meat surface by dipping or spraying has been shown to have the active agent rapidly diffuse into the bulk of the meat. Therefore, the results on surface microflora inhibition have not been completely satisfactory (Siragusa and Dickson 1992; Quintavalla and Vicini 2002).

Environmental pollution has become another issue of concern during recent years. In the thermal processing of polymers using extrusion and injection, molding may depolymerize a polymer resulting in extractable low molecular weight compound that can be easily extracted by the food or leach out to the environment. Thus, biodegradable films are more suitable than plastic films for the elaboration of antimicrobial films. Consequently, many researchers have focused on the development of biodegradable films, using mainly food protein, lipids or polysaccharides. Biopreservatives suggested for antimicrobial packing include bacteriocins such as bavaricin, brevicin, carnocin, nisin, pediocin, and lacticin and also antimicrobial enzymes such as lysozyme, lactoperoxidase, chitinase and glucose oxidase (Suppakul and others 2003). Recent studies have used edible coatings to test the effectiveness of antimicrobial agents like sorbic acid, propionic acid, potassium sorbate, benzoic acid, sodium benzoate and citric acid (Ouattara and others 2000a; Quintavalla and Vicini 2002). Bacteriocins like nisin and pediocin (Sebti and Coma 2002), enzymes such peroxidase and lysozyme (Padgett and others 1998), and also

polysaccharides with antimicrobial properties like chitosan (Ouattara and others 2000b) have also been tested.

Many polysaccharides have been used for the production of edible packaging; however, starch has been found to be an interesting material for edible films because is biodegradable, abundant, cheap and easy to use (Mali and others 2002). In 1999, a study was conducted on the properties of an edible starch-alginate film containing different concentrations of calcium ions. This study focused on the optical and mechanical properties of the film. Calcium ion concentration was directly related to mechanical strength, and inversely related to elongation properties of the film. Calcium had no effect on color, opacity, brightness and yellowness of the film. However, the presence of clots and tiny air bubbles in the cast films were observed at higher concentration of the calcium ion (Sivanandan and others 1999).

The purpose of this study was to produce a powder containing viable lactic acid bacteria cells and its metabolic products (mainly bacteriocins and lactic acid), incorporate them into an edible starch based-film and finally test their antimicrobial properties against the growth of *L. monocytogenes* in a selected RTE meat product.

Material and Methods

Inoculum

A bead containing a pure culture of *Listeria monocytogenes* Scott A, obtained from the Center of Food Safety, The University of Georgia, Griffin, GA., was activated during three days at 37°C, with two consecutives transfers between 18 to 24 h. After the last transfer and growth in TSA (TSB, Difco Laboratories, Division of Becton Dickinson and co., Sparks, Md., U.S.A.) for 18-24 h at 37°C, the culture was identified to be *Listeria* using a Micro- ID™ *Listeria* system

(Remel, Lenexa, Kans., U.S.A.). Lactic acid bacteria (*L. carnosum* 4010) selected based on results of previous experiments. The stock culture was activated in DeMan, Rogosa, Sharpe broth (MRS, Difco Laboratories, Division of Becton Dickinson and Co., Sparks, Md., U.S.A.) broth for three days, with consecutive transfers after (18 to 24 h). The culture was activated from an original stock culture of *Leuconostoc carnosum* 4010 B-SF-43 Bactoform™ CHR HANSEN.

Antimicrobial component of microbial culture

Initial experiments were conducted to determine the volume of supernatant from the bacterial culture needed to inhibit an area of TSA agar seeded with specific concentration of *Listeria*. In previous work, agar diffusion assay showed that 1 ml of supernatant was the minimum inhibitory concentration (MIC) to produce a clear zone of inhibition in agar plate (37°C for 24 h) in an area where *Listeria* was present in a concentration of ca. 100 CFU/cm². Keeping that ratio between supernatant and *Listeria* concentration, the hypothesis was that 10 ml of supernatant would be able to inhibit 1000 CFU of *Listeria* spread in an area of 25 cm² of turkey roll. Consequently, there was a requirement of 500 ml of supernatant to incorporate into a 1200 cm² antimicrobial film to expect inhibition of 1000 CFU of *Listeria*. Losses of antimicrobial component during the process were expected and a safety factor of 2 was considered. Finally, 1 liter of supernatant was the volume considered for the elaboration of each film (20 cm x 60 cm). One liter of supernatant containing the cells and its metabolic products was freeze dried and the collected powder was weighed and kept in polyethylene bags at -5°C until used for producing the cast starch films.

Effectiveness of the supernatant after freeze drying

The activity of the freeze dried supernatant of *L. carnosum* 4010 culture broth against *L. monocytogenes* was tested. One ml of active *L. carnosum* 4010 culture was added to 500 ml of fresh MRS broth and incubated at 25°C for 24 h. Cell-free supernatant was obtained after centrifugation and filtration. Lactose (20%) was added in order to increase the solid content. This liquid was poured in sterile trays covered with sterile lids and was frozen overnight at -80°C. The trays containing the frozen liquid were covered with sterile cheese cloth to avoid contamination after the freeze dry process. The trays were inserted inside a freeze dryer for 24 h; the dried supernatant (powder) was recovered and recollected in polyethylene bags.

Agar diffusion assay was used to test the effectiveness of the freeze dried supernatant. *L. monocytogenes* was pour plated in TSA agar in a concentration of 10^4 CFU/ml. After the medium had solidified, three wells were placed in each plate. Two wells were used to test the supernatant and one for control. The dried supernatant was hydrated in 500 ml of 0.1% peptone water. 1 ml was inoculated inside the well. The plates were incubated at 30°C for 24 h. Three replications were made.

Viability of lactic acid cells after freeze drying

After filtration, the bottles containing the cells were filled with 500 ml of 0.1% of peptone water and 5% of Inositol, a component to prevent death of cells, was added. This liquid containing the cells was poured inside a sterile tray covered with a lid and was frozen overnight. The trays were covered with sterile cheese cloth and they were inserted inside the freeze drier for 24 h. The dried supernatant (powder) was recollected in sterile polyethylene bags. The powder was hydrated in 500 ml of 0.1% peptone water and consecutive dilutions were made. 0.1 ml was

taken of each dilution and was spread plated in MRS agar and incubated at 25°C for 48 h. Colonies were counted. This experiment was done in triplicate.

Edible film preparation

The film forming solution was prepared in 250 ml Erlenmeyer flasks. The ingredients were added to the distilled water in the following order: sodium alginate, starch, glycerol and calcium chloride following instructions developed by (Sivanandan and others 1999). All the ingredients were mixed using a shaker (G24 Environmental Incubator Shaker; New Brunswick Scientific Edison, N.J., U.S.A.) set at 140 RPM, room temperature (22°C) until complete dissolution of ingredients. This solution was heated using a water bath (LAUDA E100; LAUDA DR. R. Wobser GMBH & Co. KG; Germany) with constant agitation for 10 min at 95 to 100°C. The mixture was poured inside the vessel of a film applicator (Thin Film applicator, DESAGA Brinkmann, Germany) Figure 3.3. The vessel was inverted to pour the solution on a PVC plate (25 cm x 65 cm). The applicator was moved forward spreading the solution evenly on the plate. The film was allowed to dry for 24 h inside a Safety Cabinet (NUAIRE™ Biological Safety Cabinet, Class II type A2, Plymouth MN, U.S.A.). Finally, the film was cut and the pieces were peeled off from the PVC plate and kept inside polyethylene bags at -5°C until use. Film # 2 and 3 from Table 3.0 were developed for this experiment based on the results reported by (Sivanandan and others 1999).

Table 3.0 Different composition for cast films.

| Film # | Cornstarch (g) | CaCl ₂ .2H ₂ O (g) | Glycerol (ml) | Sodium Alginate (g) | Distilled Water (ml) |
|--------|----------------|--|---------------|---------------------|----------------------|
| 0 | 5.500 | 0.000 | 2.000 | 0.500 | 78.000 |
| 1 | 5.495 | 0.005 | 2.000 | 0.500 | 78.000 |
| 2 | 5.490 | 0.010 | 2.000 | 0.500 | 78.000 |
| 3 | 5.480 | 0.020 | 2.000 | 0.500 | 78.000 |
| 4 | 5.470 | 0.030 | 2.000 | 0.500 | 78.000 |
| 5 | 5.460 | 0.040 | 2.000 | 0.500 | 78.000 |

*Table from (Sivanandan and others 1999).

Incorporation of the antimicrobial component

Incorporation of the antimicrobial powder into the film was done using four methods:

- Spray method after film casting:*** After the film ingredients were mixed at 90 -100°C for 10 min, the film was cast over sterile PVC plates and it was dried inside a Safety Cabinet for three days. The freeze dried antimicrobial powder was hydrated in 50 ml of sterile water and sprayed over the dried film using a capillary spray.
- Sieve method after film casting:*** the film was cast over sterile PVC plates and before it was dried, the freeze dried antimicrobial powder was added using a sterile sieve (USA Standard sieve series, Newark, New Jersey, U.S.A) with pore opening 300 µm.
- Incorporation before film casting:*** The mixture for the film was set inside a safety cabinet until the temperature dropped to 40°C. The freeze dried anti-microbial agent was added and completely dispersed using a blender for 20 seconds intervals during 1 minute. The mixture was immediately poured into the sterile applicator vessel and cast on a sterile PVC plate. The cast film was allowed to dry inside the safety cabinet for two days.
- Incorporation before film casting and use of sterile tape to maintain integrity of the film:*** The procedure was the same as described before but in this case the PVC plates were covered with sterile surgical tape (3M TM 15273 TransporeTM Clear Plastic

Perforated Surgical) before the edible film mixture was cast over it and dried for two days. Finally, the dry film was cut using sterile scalpel in 6 cm squares and placed in one of the interior sides of each zip lock bags. The bags were closed and kept at -5°C.

The best method was selected and the film was cast using the applicator vessel with three different settings for thickness: 0.8, 1.0 and 1.2 mm denoted as Film A, B, and C, respectively. Once the films were dried, the final thickness was measured. Random places of the film were selected for measurement and the average was reported.

Lactic acid bacteria recovery (*L. carnosum* 4010)

L. carnosum 4010 was recovered only from the cast film C. Areas of the film were selected and squares of 5 cm were cut and placed inside the polyethylene bags. 100 ml of 0.1% peptone water was added and stomached for 1 minute. Consecutive dilutions were made and plated on MRS agar. Three replications were made.

Agar diffusion assay

Different areas of the films were selected and were cut into 1 cm squares. The pieces were placed face down on Modified oxford plates seeded with 10^3 and 10^4 CFU/ml of *L. monocytogenes*. The inhibition zones around the squares were highlighted by the dark color of the agar in the areas that *Listeria* was able to grow. Four squares were placed per plate. Three films were antimicrobial films cast at the established thickness. The control was the sterile tape coated with the edible film without the antimicrobial agent. Plates were incubated at 30°C for 24 h. Each film was tested three times. A clear and colorless area around the films indicated inhibitory activity against *L. monocytogenes*. The test film that showed the largest inhibition zone was selected for further experiments.

Ready-to-eat (RTE) turkey rolls

Ready to eat turkey rolls from a commercial manufacturer were used for this experiment. The turkey products weighed between 4.29 kg and 4.38 kg. The turkey rolls were shipped to The University of Georgia overnight in Styrofoam boxes with polyfoam refrigerant packs. These products consisted of turkey broth, white turkey meat, modified food starch, dextrose, salt, carrageenan, and sodium phosphate. The turkey rolls were kept under refrigeration at 4°C and just before the experiment were cut into 4 sections and each section was considered as a unit for the consecutive experiment replications.

Inoculation of the turkey roll

A turkey roll unit was aseptically cut using sterile scalpel in rectangles of 25 ± 1 g; the pieces were cut using sterile templates in order to obtain areas of 25 cm^2 . One of these sides was used for the application of the inoculum. After cutting, the selected area of each piece was inoculated with 0.1 ml of *L. monocytogenes* to obtain the desired concentration. Three levels of inoculum: were established: 10^2 , 10^3 and 10^5 CFU/ 25 cm^2 . Each piece of inoculated turkey was placed inside a polyethylene bag to which the antilisterial film was attached to one of the inside surfaces. The antilisterial film was a 6 cm square. The inoculated side of the turkey was placed in contact with the antilisterial film with the turkey on top of the film. The bag was then sealed. Each bag was incubated at 4°C. Viable *L. monocytogenes* was evaluated four different times at: 0, 3, 7, and 14 days. Four control groups were used; Control 0 was the turkey roll by itself to confirm the initial zero level of *Listeria* in this product. Control 1, 2 and 3 were for analysis of the growth of three levels of *Listeria* on this type of product at the experimental conditions.

Listeria recovery from inoculated turkey roll

Aerobic plate count

100 ml of 0.1% sterile peptone was added and the bag was placed in a Stomacher for 1 min. Serial dilutions were made and 1 ml of the solution was taken and aliquots of 0.2 ml were inoculated in 5 MOX plates. The recovery was done by spread plating. The sum of the colonies found in the five plates was reported as the CFU/ml. Duplicates were made. The CFU reported was the average of the duplicates.

Listeria enrichment

Enrichment procedures were carried out for Control 0 and T₁. The USDA's *Listeria* enrichment procedure was used. This procedure requires 25 ± 1.0 g of sample; the turkey pieces were cut in order to follow this requirement. Once presumptive *Listeria* was identified after the enrichment procedures, the presumptive colonies were isolated and identified using the Micro-ID™ *Listeria* system (Remel, Lenexa, Kans, U.S.A). Results indicate presence (+) or absence (-) of *L. monocytogenes*.

Statistical Analysis

The average bacterial plate counts were transformed to logarithmic counts and analyzed for significant differences ($p < 0.05$). The minimum detection level was 100 CFU/25 cm² of turkey. In order to facilitate the statistical analysis of these data, samples with bacterial counts below the minimum detection were assigned a value of 100 CFU/25 cm². The analysis was conducted using the PROC GLM procedure in the Statistical Analysis System (SAS, 2001). Each film was analyzed separately. Each treatment was analyzed against its respective control, and a Completely Randomized Block Design was performed for testing the null hypothesis that the

mean of \log_{10} of the response variable for the treatments was the same as the control vs. the alternative that at least one mean is different.

Results and Discussions

The agar diffusion assay showed that the freeze drying process decreased the effectiveness of the supernatant. Zones of inhibition were smaller however there was complete inhibition inside the wells. The decrease in activity of the cell-free supernatant was a strong reason for the addition of viable cells. The viability of lactic acid cells after freeze drying was tested and the results showed a reduction of viable cells after the process. The average counts (\log_{10} CFU/ml) for *L. carnosum* were 10.18 before the freeze drying process and 8.37 after the process. There was an average cell reduction of 1.8 logs. Inositol was known to prevent drying of bacteria cells and protect the bacteria by cells from mechanical damage during both drying and storage (Rudge 1991). During the edible film preparation, film #2 was selected for further experiments due to the even formation of the film in contrast with film #3 which contained clumps and more air bubbles in the film. This film had double the content of calcium chloride as film #2. Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) is a highly soluble salt and tends to form clumps due to the almost instantaneous crosslink formation with the carboxyl group ($-\text{COOH}$) of the alginate. The presence of air bubbles increased with increasing levels of calcium ion because the casting solution was very viscous therefore stirring incorporated air resulting in visible bubbles in the dried film. Thus, the presence of clumps and bubbles were more pronounced in films as concentration of calcium chloride increased. However, the presence of calcium chloride was necessary to increase the film's mechanical strength (ultimate tensile strength and tear resistance). The film strength has previously shown to increase in direct proportion to the

concentration of this salt (Sivanandan and others 1999). For the incorporation of the antimicrobial powder into the film, the Spray method (a) did not result in the consistent application of the desired amount of antimicrobial agent over the film surface and spraying required a high pressure to spray the viscous liquid containing the antimicrobial powder. The sieve method (b) was also unsatisfactory in consistently applying the desired amount of antimicrobial agent over the surface, the distribution was uneven and the particles did not easily adhere to the film. Method (c) where antimicrobial powder was added to the casting solution and blending gave the best results (Figure 3.4 and 3.5). Other authors have also reported a loss of activity of *L. plantarum* cell free supernatant when it was exposed to a temperature greater than 30°C for 30 minutes (Lash and others 2005). The incorporation of antimicrobial agent was achieved when the film casting solution was at 40°C to take advantage of the low viscosity thus minimizing air incorporation when the mixture was stirred following powder addition. Although it was possible to cast the film with the antimicrobial powder, the physical properties of the original film was altered. The film became fragile; a possible solution could have been the addition of more calcium chloride; however this salt tended to form clumps and bubbles which are not desirable in the film. Consequently, sterile tape was used as a base support. Finally, the best method for incorporation of the antimicrobial powder to the film was before casting of the film and with the use of the sterile tape to maintain integrity of the film. Final thickness of the films is shown on Table 3.1. *L. carnosum* 4010 was recovered from the films and 4.72 ± 0.15 log₁₀ CFU/25 cm² was the average in the film in contact with 25 g of turkey product. Consequently, there was a concentration of ca. 10³ CFU/g of *L. carnosum* 4010 in each turkey roll analyzed. Other authors found that a concentration of 10⁶ CFU/g of *L. carnosum* 4010 was necessary to inhibit a cocktail culture of *Listeria* at 10⁴CFU/g during storage conditions at 5°C

for 21 days (Budde and others 2003). Figure 3.6 shows the activity of the antimicrobial films against *L. monocytogenes*. Agar diffusion assay identified two films with larger inhibition zones. Antimicrobial films B and C with thickness: 0.21 and 0.51 mm respectively were selected for application on turkey rolls.

Recovery of *L. monocytogenes* after 0 days storage

Table 3.2 at day 0 shows viable cell counts of *L. monocytogenes* after inoculation. Each control contained the target inoculum. The results showed that counts for all the treatments were at the same level as their respective controls. The bacterial action of LAB cells and their metabolic products were not immediate. *Listeria* enrichment for control 0 was negative, confirming undetectable levels of *L. monocytogenes* in the fresh turkey rolls before the experiments.

Recovery of *L. monocytogenes* after 3 days storage

Film C showed a slightly reduction of *Listeria*; however, Film B didn't show any antimicrobial effect, the counts for the different inoculation levels were the same as the ones on the Controls. The enrichment procedure resulted in positive recovery for *L. monocytogenes* in Treatment 1 (Treatment with the lowest inoculation of *Listeria*), see Table 3.2.

Recovery of *L. monocytogenes* after 7 days storage

Film B showed a slight reduction in the viable counts. Control 2 originally contained 4.16 ± 0.09 (\log_{10} CFU/25 cm² \pm S.D) and the counts after the treatment with film B were 3.70 ± 0.18 \log_{10} CFU/25 cm² \pm S.D. For the treatment with the higher inoculation level (T3), Film B showed a bacteriostatic effect (Figure 3.1). On the other hand, Film C showed reduction in both treatments with the high inoculation levels (T2 and T3), Control 3 contained 5.62 ± 0.03 (\log_{10}

CFU/25 cm² \pm S.D) and T3 was $5.04 \pm 0.15 \log_{10}$ CFU/25 cm² \pm S.D.; a 0.6 log₁₀ CFU reduction. However, *Listeria* was still recovered in T1 (Table 3.2 and Figure 3.2).

Recovery of *L. monocytogenes* at 14 days storage

The growth of *L. monocytogenes* in all the inoculated controls was evidenced by the end of the second week of storage. About one log₁₀ CFU was the increment reached compared with the one reported at 0 day storage. No CFU were enumerated at the lowest inoculation level (T1) for Film C; however, turkey rolls were found to be positive for *L. monocytogenes* after *Listeria* enrichment procedures. The absence of *Listeria* colonies during plate counting showed that Film C had *Listeria* inhibitory potential but there were still survivors or injured cells in less than 100 CFU per piece of turkey roll. *Listeria* enrichment showed the presence of *Listeria* after treatment with both films. The effect of film B was noticeable only at the treatment with second level of *Listeria* inoculation (T2). The original counts for control 2 after 14 days were $5.01 \pm 0.33 \log_{10}$ CFU/25 cm² \pm S.D. and the counts after treatment (Film B) were $4.42 \pm 0.61 \log_{10}$ CFU/25 cm² \pm S.D., showing a 0.6 log₁₀ CFU reduction. On the other hand, Film C reached higher levels of inhibition: 1.84 log₁₀ CFU and 1.54 log₁₀ CFU for initial *Listeria* inoculation levels of 10³ CFU/25 cm² and 10⁵ CFU/25 cm², respectively. The reported reduction was in relation with their respective controls (Table 3.2 and Figure 3.2). The statistical analysis showed that film B achieved significant *Listeria* reduction ($p < 0.05$) for levels of ca. 10² CFU/25 cm² and 10³ CFU/ 25 cm² compared with their respective controls for day 7 and 14. No significant reduction ($p > 0.05$) was achieved with the highest level of *Listeria* (10⁵ CFU/25 cm²). On the other hand, Film C achieved significant reduction at the three levels of *Listeria*. For level ca. 10² CFU/25 cm² a significant reduction was achieved from day 0 until day 14, while for the second level ca. 10³ CFU/25 cm², reduction was significant for day 7 and 14. Finally, the film showed

significant reduction at the highest *Listeria* inoculation (ca. 10^5 CFU/25 cm²) from day 3 until day 14. In order to compare both films, the controls done in different days for each film were analyzed for significant differences. The results showed no significant difference, consequently Film B was compared with Film C.

Conclusions

Antimicrobial films containing LAB cells and their metabolic products seemed to be a promising option for the RTE meat industry. The best method of incorporation of the antimicrobial powder was before casting the film at 40°C and with the use of a blender. Of the three thickness of film developed, Film C with 0.51 ± 0.02 mm proved to have major effectiveness in inhibiting *Listeria monocytogenes* Scott A in a real food system. Film C was better than Film B. Complete inhibition was not achieved; however, significant reduction (almost $2 \log_{10}$ CFU/25 cm²) was achieved after 14 days of treatment when initial *Listeria* inoculation was 10^3 CFU/25 cm².

Table 3.1 Final thickness of films B and C (Applicator vessel settings: 1.0 and 1.2 mm, respectively).

| Film B | Antimicrobial Powder (g) | Thickness of Film (mm) | | | Average (mm) | Sterile Tape (mm) | Thickness Antimicrobial film (mm) |
|--------|--------------------------|------------------------|-------|-------|--------------|-------------------|-----------------------------------|
| Rep 1 | 98.24 | 0.330 | 0.381 | 0.342 | 0.338 | 0.15 | 0.188 |
| Rep 2 | 99.36 | 0.355 | 0.381 | 0.368 | 0.364 | 0.15 | 0.214 |
| Rep 3 | 102.65 | 0.406 | 0.368 | 0.381 | 0.389 | 0.15 | 0.239 |
| | | | | | | | Film B: 0.21 ± 0.03 |

| Film C | Antimicrobial Powder (g) | Thickness of Film (mm) | | | Average (mm) | Sterile Tape (mm) | Thickness Antimicrobial film (mm) |
|--------|--------------------------|------------------------|-------|-------|--------------|-------------------|-----------------------------------|
| Rep 1 | 96.91 | 0.635 | 0.622 | 0.647 | 0.643 | 0.15 | 0.493 |
| Rep 2 | 103.70 | 0.660 | 0.635 | 0.647 | 0.651 | 0.15 | 0.501 |
| Rep 3 | 101.04 | 0.673 | 0.660 | 0.685 | 0.681 | 0.15 | 0.531 |
| | | | | | | | Film C: 0.51 ± 0.02 |

Table 3.2 Viable cells (\log_{10} CFU/25 cm² ± S.D.) of *L. monocytogenes* at 0, 3, 7 and 14 days in turkey roll after antimicrobial film application.

| Controls & Treatments | Day 0 | | Day 3 | |
|-----------------------|------------------|------------------|------------------|------------------|
| | Film B (0.21 mm) | Film C (0.51 mm) | Film B (0.21 mm) | Film C (0.51 mm) |
| | | | | |
| Control 0 | b | b | b | b |
| Control 1 | 2.41 ± 0.09 | 2.57 ± 0.05e | 2.31 ± 0.11 | 2.45 ± 0.15e |
| Control 2 | 3.49 ± 0.16 | 3.49 ± 0.07 | 3.61 ± 0.05 | 3.56 ± 0.10 |
| Control 3 | 5.32 ± 0.11 | 5.29 ± 0.18 | 5.26 ± 0.21 | 5.51 ± 0.10g |
| T 1 | 2.25 ± 0.15 | 2.33 ± 0.11e | < 2.00a | < 2.00ae |
| T 2 | 3.60 ± 0.10 | 3.43 ± 0.09 | 3.50 ± 0.09 | 3.37 ± 0.08 |
| T 3 | 5.41 ± 0.03 | 5.13 ± 0.15 | 5.41 ± 0.11 | 5.27 ± 0.11g |
| Controls & Treatments | Day 7 | | Day 14 | |
| | Film B (0.21 mm) | Film C (0.51 mm) | Film B (0.21 mm) | Film C (0.51 mm) |
| | | | | |
| Control 0 | b | b | b | b |
| Control 1 | 2.56 ± 0.11c | 2.72 ± 0.11e | 3.34 ± 0.11c | 3.51 ± 0.19e |
| Control 2 | 4.16 ± 0.09d | 3.84 ± 0.15f | 5.01 ± 0.33d | 4.57 ± 0.21f |
| Control 3 | 5.45 ± 0.05 | 5.62 ± 0.03g | 6.42 ± 0.05 | 6.45 ± 0.23g |
| T 1 | < 2.00ac | < 2.00ae | < 2.00ac | < 2.00ae |
| T 2 | 3.70 ± 0.18d | 3.23 ± 0.19f | 4.42 ± 0.61d | 2.73 ± 0.12f |
| T 3 | 5.56 ± 0.11 | 5.04 ± 0.15g | 6.31 ± 0.52 | 4.91 ± 0.28g |

a Positive after USDA's *Listeria* enrichment.

b Negative after USDA's *Listeria* enrichment.

c,d,e,f,g repeated letters in the same column are significantly different ($p < 0.05$)

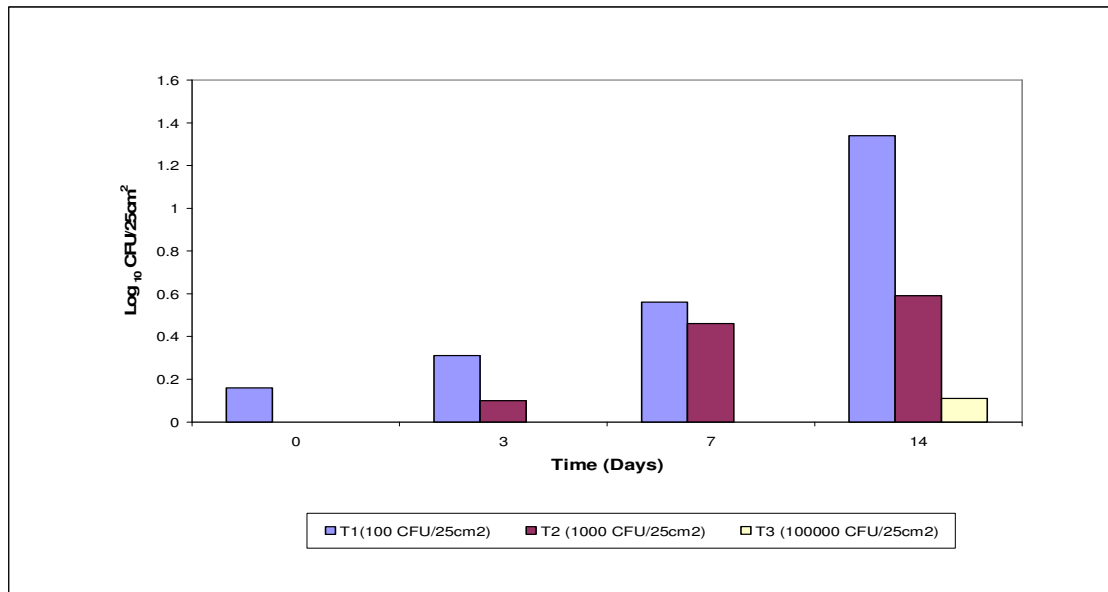


Figure 3.1 Antimicrobial FILM B: Achieved reduction of *L. monocytogenes* (log₁₀ CFU/25 cm²) at 0, 3, 7 and 14 days of treatment.

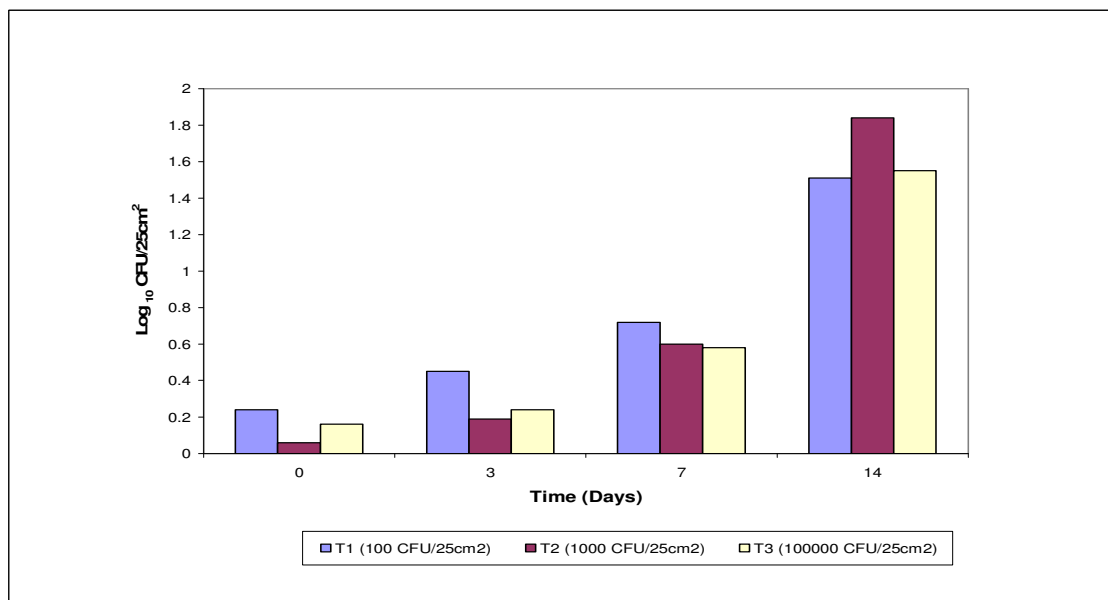


Figure 3.2 Antimicrobial FILM C: Achieved reduction of *L. monocytogenes* (log₁₀ CFU/25 cm²) at 0, 3, 7 and 14 days of treatment.

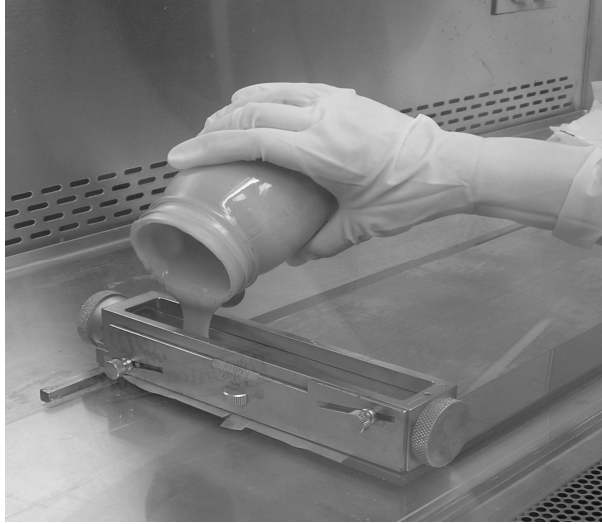


Figure 3.3 Applicator vessel on top of PVC plate.

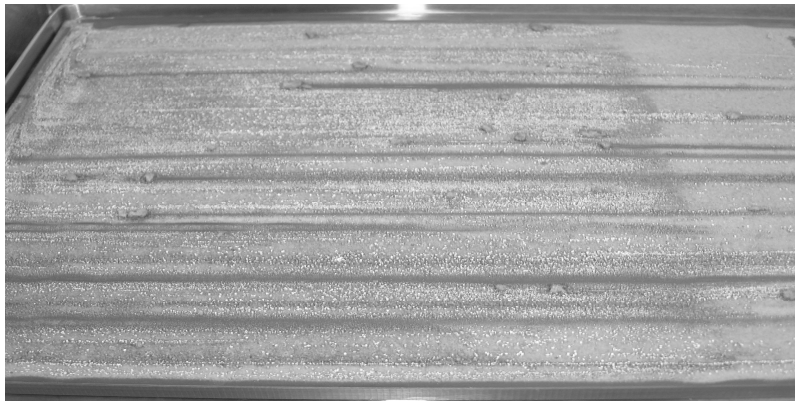


Figure 3.4 Antimicrobial film – powder manually mixed.

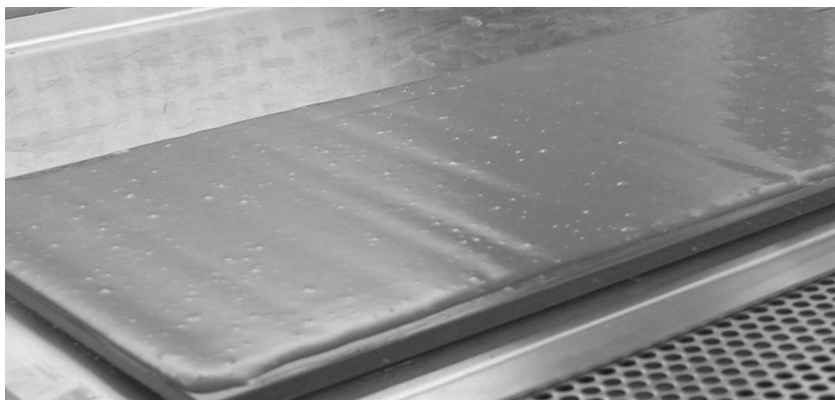


Figure 3.5 Antimicrobial film – powder incorporated with a blender.

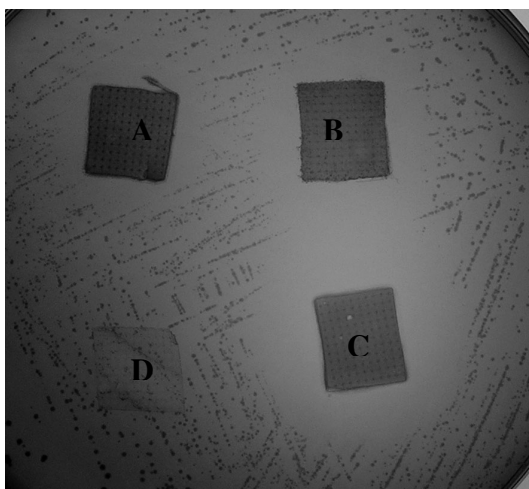


Figure 3.6 Agar diffusion assay: *L. carnosum* 4010 – coated film against *L. monocytogenes*.

* Thickness: Film A: 0.12mm; Film B: 0.21mm; Film C: 0.51mm; and Film D: control.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The food safety hazard of *Listeria* in ready to eat (RTE) meat products has called attention to the potential use of some lactic acid bacteria (LAB) that can inhibit spoilage bacteria and pathogens such as *Listeria monocytogenes* (LM). RTE meat products inoculated with LAB can be made safe if the LAB produces large amounts of organic acids. However, too much acid production will alter the product's flavor and possibly texture. Consequently, selection of a suitable LAB is a very important part of the process of adopting this biotechnological approach of improving RTE meat safety. From the three meat-borne LAB tested, *L. carnosum* 4010 was shown to produce more active bacteriocins with a lower lactic acid production compared to *L. carnosum* ATCC 49367 and *L. plantarum* ATCC 8014.

It is traditionally believed that bacteriocins are mainly responsible for the initial inhibitory effect of LAB culture medium. However, the agar diffusion assay indicated that there is a synergistic effect between bacteriocin and lactic acid. Finally, live LAB cells exhibit the additional inhibitory effect only after 21 days of incubation at 4°C. Eventually LAB cells proliferate and inhibit LM through competitive inhibition and additional production of bacteriocins and lactic acid.

The minimum inhibitory concentration (MIC) in a planktonic environment was found to be 8 ml of cell-free supernatant for 1 ml of *Listeria* at ca. 10^3 cfu/ml. Agar diffusion assay showed a MIC of 1 ml of cell-free supernatant for an area of seeded agar with *Listeria* ca. 100 CFU/cm². Better inhibition was achieved when pH of the LAB growing medium was modified close to 4.0. Treatment of the LAB culture medium with proteolytic enzymes demonstrated that the three LAB tested produced a bacteriocin which is a protein in nature. LAB with potent bacteriocin and low lactic acid production are strong candidates to use as protective cultures to

meet the increasing consumer demand for less processed foods and to comply with the USDA final rule 9CFR430.4 of employing growth inhibitors for *Listeria* after lethality treatment.

Listeria contamination of RTE meat products tend to occur on the surface during post-processing handling. Therefore, the application of the antimicrobial on an edible film as antimicrobial packaging is very promising. Antibacterial films were developed utilizing *Leuconostoc carnosum* 4010 as the antimicrobial component (cells and metabolic products). The best method of antimicrobial incorporation was found to be before film casting at 40°C where the dried LAB culture medium was incorporated using a blender. The use of sterile tape as a base was necessary to maintain the film integrity. Two thickness of films: B (0.21 ± 0.03 mm) and C (0.51 ± 0.02 mm) showed the largest inhibition zones in agar diffusion assay. Although the antimicrobial film did not inactivate cells of *L. monocytogenes*, it prevented growth and eventually reduced the number of colony forming units. After the 14 days treatment *L. monocytogenes* was positive after enrichment from samples with inoculation levels of *Listeria* (ca. 100 cfu/25 cm²) regardless of the type of film used. However, Film C produced the highest degree of inhibition after 14 days of treatment; almost 2 log₁₀ CFU/ 25 cm² and 1.5 log₁₀ CFU/25 cm² reduction of *Listeria* when the initial inoculation was 10³ CFU/25 cm² and 10⁵ CFU/25 cm², respectively.