INTRODUCTION

*Listeria monocytogenes* presents a serious threat to consumer health and safety and has been implicated in several deadly outbreaks in the United States and around the world. The USDA and FDA are both currently enforcing a "zero tolerance" policy for *L. monocytogenes* in ready-to-eat foods in the United States. This organism is halotolerant, resistant to freezing temperatures, and can grow and multiply during refrigeration, where other competing organisms cannot. Because of the pervasive nature of *L. monocytogenes*, it is difficult for processors to fully eliminate the organism from the environment.

In storage of fresh foods, such as raw meat or fish, pathogens are normally of lesser concern since the Gram-negative spoilage bacteria present grow much more rapidly (Jeppesen and Huss, 1993). Under temperature abuse, spoilage of the product occurs before pathogens can proliferate. However, in ready-to-eat products, cooking, preservation ingredients, and storage atmosphere inhibit the native Gram-negative organisms, resulting in a longer shelf life. This enhances the growth conditions of psychotropic pathogens such as *Listeria*, allowing them to grow to dangerous levels (Francis and O'Beirne, 1998). Because lactic acid bacteria can grow under the same storage conditions as *Listeria spp*., many studies have been conducted to investigate if these Gram-positive organisms can provide adequate competition against the pathogenic organisms also present.

Ready-to-eat meat products such as deli meats and frankfurters are at a particularly high risk to consumers, as these products may be heated improperly or recontaminated prior to consumption. Recycled brines are often used to cool thermally

processed frankfurters and deli meats. This rapid cooling prevents the growth of many pathogenic organisms that survive the initial heat treatment. Heat and nutrients are transferred to the brines during the chilling process, increasing the risk of bacterial contamination. Because *L. monocytogenes* can survive at refrigeration temperatures and high salt concentrations (up to 28% w/v for short periods), a treatment is necessary to prevent food-borne illness from contaminated brines.

Various species of lactic acid bacteria (LAB) present during production are the major contributors to spoilage of frankfurters. Both *L. monocytogenes* and lactic acid bacteria can thrive under similar conditions. This competition for resources has the capability to reduce the number of pathogenic organisms. Studies performed on various meat products have shown that inoculation with strains of LAB can inhibit the growth of *L. monocytogenes*. Because the infectious dose of *L. monocytogenes* is not known, it is necessary to reduce or inhibit this pathogen as much as possible to ensure the safety of the product. The ability of three LAB strains to reduce *L. monocytogenes* in brine was investigated. The response of *L. innocua* to the same conditions was also investigated as a possible useful nonpathogenic model.

LITERATURE REVIEW

## A. *Listeria monocytogenes*

### 1. Organism characteristics

*Listeria monocytogenes* is a common foodborne pathogen that is ubiquitous in nature. It may be found in plants, soil, or water, and has been isolated from many mammalian and bird species. *Listeria monocytogenes* is a short, Gram-positive, non-sporeforming rod, with tumbling end-over-end motility at room temperature (Brooks et al, 1998). It is a catalase positive, oxidase negative, facultative anaerobe with slight β–hemolysis on blood agar. *Listeria monocytogenes* has been known to survive refrigeration, freezing, heating, and drying, which creates obstacles for the food industry (CFSAN, 2001). It has optimum growth at 32-35°C, but can survive and multiply at refrigeration temperatures. It has been found in raw foods, such as fruits, vegetables, and uncooked meats, and has been associated with outbreaks in raw milk, ice cream, raw meats, and ready-to-eat (RTE) meat and cheese products.

### 2. Disease characteristics

*Listeria monocytogenes* is responsible for approximately 2,500 illnesses and 500 deaths in the United States each year (CDC, 2000). Most healthy adults have few or no symptoms, as the disease generally affects those with compromised immune systems. In at-risk populations, flu-like symptoms including fever, headache, nausea, vomiting, and diarrhea appear about 12 hours or more after ingestion. After several days, the more serious symptoms appear, including meningitis, encephalitis, septicemia, and intrauterine/cervical infections that may result in spontaneous abortion in pregnant

women (CFSAN, 2001). Generally, mortality rates for listeriosis may be as high as 80% for neonatal infections, and 50-70% for meningitis and septicemia patients (CFSAN, 2001). The infective dose of *L. monocytogenes* is currently unknown, although it appears to be above 100 viable cells, depending on pathogen strain and susceptibility of the host (Roberts, 1994).

## 3. Foodborne outbreaks

While pasteurization and cooking methods used by processors can kill *Listeria monocytogenes*, post-processing contamination may occur because the organism is so resilient in the environment. The largest meat recall in history occurred in October 2002, when 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken products were recalled after a multi-state listeriosis outbreak. Eight states reported a total of 53 culture-confirmed cases, all in immunocompromised individuals, resulting in eight deaths and three stillbirths or miscarriages (CDC, 2002). In November 2000, twelve cases of *L. monocytogenes* infection were reported in a Hispanic community in North Carolina after consumption of homemade Mexican-style soft cheese. Ten of these cases were in pregnant women, resulting in five stillbirths, three premature deliveries, and two infected newborns (CDC, 2000). From May to November of the same year, ten states reported 29 cases of listeriosis from deli turkey meat, resulting in four deaths and three stillbirths or miscarriages (CDC, 2001). In late 1998, over 50 illnesses were linked to a rare strain of *L. monocytogenes* that was found in a large frankfurter and deli meat plant (CDC, 1998). Six deaths and two miscarriages resulted in the ten states affected. One of the earlier outbreaks was in California in early 1985, where 86 cases, 16 deaths, and 13 stillbirths

were linked to a Hispanic community and Mexican-style soft cheese. Sixty-three percent of these cases were among mother-newborn pairs (CDC, 1985).

## 4. Survival in ready-to-eat meat products

### a. Temperature

*Listeria monocytogenes* is capable of survival and growth over a wide range of storage temperatures. While the optimum growth temperature is 30-37°C, lower and upper limits for growth are 1°C and 45°C, respectively (Seeliger and Jones, 1986). Growth has also been observed in chicken broth at temperatures as low as –0.1°C to –0.4°C (Walker et al., 1990). Because of this resilience, *L. monocytogenes* has been found to survive and multiply in refrigerated processed meat products, including ham, bologna, frankfurters, sliced turkey and chicken, and sausages stored at 4.4°C (Glass and Doyle, 1989). Another study performed on frankfurters alone found 65.6% of inoculated samples stored at 5°C under vacuum for 28 days supported growth of *L. monocytogenes* (McKellar et al., 1994).

Although the lower growth limit of *L. monocytogenes* has been estimated to be around 0°C, it has been found to survive lower temperature extremes for extended periods of time. In ground turkey and frankfurters inoculated with the pathogen, only a 1 to 3 log reduction was obtained after storage at –18°C for 8 weeks (Palumbo and Williams, 1989). The organism has also been shown to survive at temperatures in excess of its upper growth limit, 45°C. A study by Novak and Juneja (2003) found that heat-shocking *L. monocytogenes* in ground beef at 46°C for 60 minutes caused $D_{10}$-values to increase 1.4 fold when compared to non-shocked controls. Further

storage under refrigerated conditions did not change this heat-resistance. Because of the temperature fluctuation that occurs during the display, purchase, transfer, and storage of a refrigerated food product, there is a high risk of increasing pathogen populations.

**b. Sodium chloride (NaCl) concentration**

*Listeria monocytogenes* is halotolerant, resistant to the inhibitory effects of sodium chloride. High amounts of salt must be employed in order to prevent the pathogen from proliferating. McClure et al. (1989) observed growth of *L. monocytogenes* within 72 hours in 10% NaCl at 25°C. Peterson et al. (1993) investigated sodium chloride and packaging method as a means to control contamination of cold-smoked salmon. Greatest inhibition of *L. monocytogenes* was observed in 6% NaCl at 5°C, reaching a level of $10^2$ cfu/g compared to the $10^6$-$10^8$ cfu/g control. Hudson (1992) found *L. monocytogenes* survived 6 hours at 10°C in solutions containing 6, 16, or 26% NaCl. *L. monocytogenes* actually grew in the 6% brine solution and survived over a month in the 16% solution. The presence of salt also partially protects *L. monocytogenes* from other stresses, such as heat. Linton et al. (1995) found that increasing NaCl concentration from 0 to 4% in an inoculated infant formula increased the heat resistance of the pathogen.

Other studies have focused on salt concentration acting as a protective cell mechanism. Faleiro et al. (2003) demonstrated that an exposure to low levels of sodium chloride protects *Listeria monocytogenes* against acid shock up to pH 3.5. The acid tolerant response also cross-protected against osmotic shock, up to 20%

NaCl. Bal'a and Marshall (1996) found *L. monocytogenes* has increased ability to tolerate higher salt concentrations and lower pH at lower incubation temperatures. Low sodium chloride concentration (2 to 3.5%) has also been shown to have a protective effect against the food preservative nisin at low temperatures (DeMartinis et al., 1997).

While salt concentration at low temperatures has a protective effect on the survival of *L. monocytogenes*, it may be utilized in conjunction with other stress factors for pathogen reduction. Buchanan et al. (1989) studied the interaction of various temperatures, pH, atmosphere, sodium chloride content, and sodium nitrate content on the growth of *L. monocytogenes* strain Scott A. While sodium nitrite alone had little effect on growth, a combination of anaerobic conditions, 4.5% NaCl, and pH 6.0 notably increased the effectiveness of sodium nitrite. Vialette et al. (2003) found the presence of salt (8%) induced longer lag phase and increased the generation time of clinical and food isolates of *L. monocytogenes* by an average factor of 2.5 when compared to control. These studies demonstrate that while this pathogen is resistant to many environments, *L. monocytogenes* may be susceptible to cell injury and death under appropriate conditions.

**c. Water activity**

*Listeria monocytogenes* is able to multiply in many foods because it can survive and thrive at low water activity ($a_w$). A study using 39.4% sucrose solution observed the growth of *L. monocytogenes* at the minimum $a_w$ of 0.92 (Petran and Zottolla, 1989). Miller (1992) found that not only is the water activity a factor in the

survival, but the type of solute used as well. *Listeria monocytogenes* Scott A was grown in brain heart infusion broth (BHI) modified to 0.99 – 0.80 $a_w$ with three solutes, yielding minimum $a_w$ for growth at 0.90 with glycerol, 0.92 with NaCl, and 0.97 with propylene glycol. Below these minimum $a_w$ levels, cell death was proportionate to the water activity. Cells died earliest in the propylene glycol, lived intermediately in NaCl, and lived longest in glycerol. Nolan et al. (1992) observed similar results in tryptic soy broth containing 0.6% yeast extract (TSBYE) using glycerol, NaCl, and sucrose. The sucrose broth had a minimum $a_w$ of 0.92. These results were again confirmed by Farber et al. (1992) using tryptic soy broth and sucrose for an observed minimum $a_w$ of 0.92.

A study using a model meat system considered the relationship of water activity and other factors on the growth of *L. monocytogenes* Scott A (Chen and Shelef, 1992). Inhibitory effects were observed in cooked strain beef at a moisture content of 25% and a water activity of 0.93 with no added solutes.

**d. pH**

Another important consideration to the growth and survival of *L. monocytogenes* is pH. The organism has a possible range of growth from pH 4.1 to 9.6, with an optimum range of 6.0-8.0 (Jay, 2000b). This pH range is dependent upon various factors, including incubation temperature, available nutrients, moisture content, and product composition. Parish and Higgins (1989) found that lower pH had a deleterious effect on viability of *L. monocytogenes*, although a lag period occurred at 4°C before cell reduction occurred. They concluded that low pH products

were of concern in *L. monocytogenes* outbreaks under contamination followed by consumption of the product soon after purchase, as the lag effect would prevent immediate cell death. Dykes and Moorhead (2000) studied the effects of acid stress on strains of *L. monocytogenes* from clinical or meat origin. After exposure to conditions of severe acid stress (pH 2.5) for 2 hours, only two strains of meat origin displayed significant reduction in number. No strains of clinical origin were affected, indicating the importance of acid stress resistance in the infection process and virulence of *Listeria monocytogenes*.

Studies previously mentioned observed a synergistic relationship between acid and osmotic shock responses in *L. monocytogenes* (Vialette et al., 2003) (Faleiro et al., 2003). Le Marc et al. (2002) also researched a combination of factors to find the growth limits of *L. monocytogenes*, including pH and organic acid concentration at set temperatures. They observed combinations of low pH, high concentrations of weak organic acid, and low temperatures were effective inhibitors of *Listeria*.

Because most meat products fall in a pH range of 5.1 – 6.4, depending on the animal of origin and the way the meat is processed (Jay, 2000b), contamination with *L. monocytogenes* and subsequent risks of illness from eating ready-to-eat meats are of major concern to food safety experts.

**e. Microbial competition**

Cooked products exposed to post-processing contamination with *L. monocytogenes* are also likely to contain Gram-negative psychrotrophic spoilage organisms. These organisms compete for available nutrients and thus influence

product safety and stability through storage (Lawlor, 1999). Examples of these spoilage organisms are the *Pseudomonas* species, which are usually found in aerobically stored meat products. These aerobic psychrotrophs usually out-compete *L. monocytogenes*, preventing the pathogen growth that can infect compromised individuals. However, if the storage atmosphere is modified, the aerobic *Pseudomonas* may be inhibited significantly, allowing *L. monocytogenes* to proliferate to infectious levels before any sign of spoilage. Marshall et al. (1991) found this result in a study on cooked chicken nuggets. Francis and O'Beirne (1998) also found increased levels of *L. monocytogenes* stored under modified atmosphere packages of vegetables due to decreased numbers of the naturally competitive microflora. Depending on storage conditions, this effect can be reversed. Buchanan and Bagi (1999) observed *Pseudomonas fluorescens* inhibition of *L. monocytogenes* at low incubation temperatures (4°C) and low sodium chloride (5 and 25 g/L). Conversely, there was also a slight increase of *L. monocytogenes* at higher temperature (12°C, 19°C) and salt concentrations (25 and 45 g/L).

Lactic acid bacteria (LAB) are also able to compete against *L. monocytogenes* within a food system. These organisms include the pediococci, lactobacilli, leuconostocs, and enterococci, which compete through the production of organic acid and bacteriocins. Lactic acid bacteria compete by lowering pH of the medium to levels unfavorable for *Listeria* and other pathogens. Organic acids, such as the lactic and acetic acids produced by LAB, have been shown to be considerably more inhibitory to *Listeria* than inorganic acids, such as hydrochloric (Farber et al., 1989). Lactic acid bacteria may also contain bacteriocins, antimicrobial proteins/peptides

produced by some microorganisms with the capability to inhibit closely related organisms (McCormick et al. 1998). Because both *Listeria* and LAB are halotolerant, psychrotrophic, and facultatively anaerobic, the bacteriocins produced by some LAB may be effective against *Listeria*.

Yousef et al. (1991) demonstrated the efficacy of pediococci and their associated bacteriocin, or pediocin, for controlling *L. monocytogenes* in wieners. This study was confirmed by Degnan et al. (1992) who observed an inhibition of *L. monocytogenes* by a bacteriocinogenic pediococci strain when grown together in vacuum-packaged wieners. Buchanan and Bagi (1997) observed suppressed growth of *L. monocytogenes* in the presence of four strains of *Carnobacterium piscicola*, two with bacteriocin ability, and two without. This was significant in demonstrating the antilisterial effects of some lactic acid bacteria without the need for a separate bacterocin. These effects are useful since many LAB cannot produce bacteriocin at refrigeration temperatures, where *L. monocytogenes* may become an issue in ready-to-eat foods.

Amézquita and Brashears (2002) screened 49 strains of lactic acid bacteria for inhibitory ability against *L. monocytogenes*. Three were chosen (*Pediococcus acidilactici, Lactobacillus casei,* and *Lactobacillus paracasei*) based on their high activity against *L. monocytogenes* at low temperatures. After 7 days of refrigerated storage, these lactic acid bacteria caused a 2 $\log_{10}$ reduction of *L. monocytogenes* in frankfurters. After 28 days of refrigerated storage, these lactic acid bacteria caused a 4.2-4.7 $\log_{10}$ reduction of *L. monocytogenes*. Further study also indicated no impact on product quality up to 56 days. This was extremely promising for increasing

product safety without decreasing shelf life or causing sensory change that may affect product quality. Giménez and Dalgaard (2004) also observed significant inhibition (5-log) of *L. monocytogenes* stored in cold-smoked salmon for 9 days with a cocktail of 10 spoilage organisms (lactic acid bacteria, enterococci), again significant to prove that inhibition is possible under low temperatures.

## B. *Listeria innocua*

*Listeria innocua* is a Gram-positive bacterial strain closely related to *Listeria monocytogenes*. The apparent difference between the species is the lack of pathogenicity of *L. innocua*. There have been over 1000 worldwide cases of human foodborne illness associated with *L. monocytogenes* in the last 35 years (CFSAN, 2001). In comparison, there had never been a case of human illness associated with *L. innocua* until recently. Perrin et al. (2003) reported a fatal case of bacteremia in a 62-year old patient in France. Regardless of this isolated case, *L. innocua* itself does not currently pose a foodborne health concern for consumers. However, the presence of *L. innocua* indicates the potential for *L. monocytogenes* contamination, which is very much of a concern for consumers and processors. The similarity between these species makes it difficult to distinguish between them, which can also be concerning to processors.

As for many other bacteria, the practical identification of *Listeria* species is based on a limited number of biochemical markers (Rocourt, 1994). Like *L. monocytogenes*, it is a Gram-positive, non-sporeforming short rod, that is catalase positive, oxidase negative, and facultatively anaerobic (Brooks et al., 1998). Unlike

*L. monocytogenes* however, *L. innocua* does not produce β-hemolysis on blood agar (Poysky et al., 1993). This characteristic has been one of the few tests available for the differentiation of *Listeria* species. Another difference is the presence of arylamidase in *L. innocua*, where as *L. monocytogenes* does not contain this substance. A mass-produced test kit, the API *Listeria* strip, uses a test for arylamidase (DIM test) as the main difference between these two *Listeria* species. Bille et al. (1992) evaluated the API strip for identification accuracy, and found that it was accurate 99.4% of the time for *L. innocua* and 97.7% of the time for *L. monocytogenes*.

Reports of *Listeria* isolation and subsequent identification in foods have shown *L. innocua* to be the most frequently isolated, followed closely by *L. monocytogenes* (Johnson et al., 1990). These reports have been repeated by several different groups of researchers (Grau and Vanderlinde, 1992; Petran and Swanson 1993; Jay 1996; Walsh et al., 1998). Given the similarities in these organisms, it is no surprise to find them together frequently in both nature and in food products. DNA homology and 16S rRNA sequencing of the *Listeria* genus have indicated the genus has 2 lines of descent (Rocourt, 1994), of which *L. innocua* and *L. monocytogenes* occupy the same line. The sequencing of the 16S rRNA was done using reverse transcriptase. Although differences vary amongst individual strains, there appears to be only 2 of 1,281 base pairs that always differ between *L. innocua* and *L. monocytogenes* consistently (Czajka et al., 1993). Small differences also occur in the V2 and V9 regions of the genotype, but these differences do not occur in every strain of *L. innocua* and *L. monocytogenes*. Buchrieser et al. (2003) compared *L. innocua*

and *L. monocytogenes* at a genetic level, and found distinct base pair content, specifically among guanine-cytosine (G-C) pairs, that suggested lateral gene transfer may be responsible for the evolution and differences in pathogenicity of the two strains. Another difference at the genetic level was documented recently by Cabanes et al. (2004). This group identified a gene (aut) encoding for a surface protein of *L. monocytogenes* absent in *L. innocua*. The protein, Auto, was shown necessary for entry of *L. monocytogenes* into eukaryotic cells, which was assumed to be indicative of a role in bacterial virulence.

One of the more interesting aspects of the *L. innocua* strain is the frequency it is used to model the behavior of *L. monocytogenes*. The ubiquitous nature of *L. monocytogenes* makes it nearly impossible to completely eradicate from an environment, thus many researchers and processors are understandably hesitant to introduce a pathogen into their working environment for fear of future contamination problems. In some cases, *L. innocua* models the behavior of *L. monocytogenes* well, as in the case of Dykes et al. (2003) who tested and compared both species of *Listeria* in cooked tiger prawns. Several other recently published studies have used *L. innocua* as a model for the pathogen, without explicitly testing the model being used (Scannel et al., 2001; Benech et al., 2002; Sommers et al., 2002; Olasupo et al., 2004).

Making generalized conclusions based on an incorrect model can cause problems if the two species do not, in fact, behave similarly. Meylheuc et al. (2002) illustrated this effect when comparing growth kinetics and physicochemical surface properties of *L. innocua* to those of *L. monocytogenes*. They found significant

difference in the physicochemical surface properties, electronegative character (particularly at 20°C), and Lewis acid-base characteristics of *L. innocua* as a function of temperature and growth stage. These differences indicated the model was inappropriate; the use of *L. innocua* was not suitable for simulating the bioadhesive behavior of pathogenic *L. monocytogenes* under the set experimental conditions. A study by Antunes et al. (2002) found different percentages of antimicrobial resistance in *L. monocytogenes* (73%) and other *Listeria* species (84%), suggesting the behavior of the pathogen would differ from a model using a non-pathogenic strain. Because *Listeria monocytogenes* has a low infectious dose and a high mortality rate, models using other non-pathogenic *Listeria* strains must be verified accordingly to ensure efficient elimination of the dangerous pathogen.

## C. Lactic Acid Bacteria

### 1. Organism characteristics

The lactic acid bacteria (LAB) are non-sporeforming rods or cocci, aerobic to facultatively anaerobic, lacking cytochromes, unable to reduce nitrates, unable to utilize lactate, oxidase-negative, catalase-negative, benzidine-negative, and gelatinase-negative (Carr et al., 2002). They are grouped together because of their common ability to ferment glucose into lactic acid. The by-products of this glucose fermentation further classify them into homo- or hetero-fermenters. Both groups produce lactic acid as the major product, but the hetero-fermenters also produce acetic acid, ethanol, and carbon dioxide (Ricke et al., 2001). The group consists of the genera *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus,*

*Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Jay, 2000a).

Lactic acid bacteria are widely used as starter cultures in the food industry: the fermentation of dairy products such as yogurt and cheese (Jay, 2000a), the malolactic fermentation of wines (Kosseva and Kennedy, 2004), and the fermentation of a variety of meat products, such as sausages (Nordal and Slinde, 1980). The lactic acid and other by-products produced by these bacteria create favorable flavors in these foods. These unique bacteria are also used in the production of other common foods, including but not limited to: pickles, olives, coffee, chocolate, and soy sauce.

Lactic acid bacteria can also be used as protective cultures to compete against potential pathogens and other undesired organisms, thereby increasing the safety of the food product. Unlike starter cultures, protective cultures should alter the sensory properties of the product as little as possible (Schillinger et al., 1991). Lactic acid bacteria inhibit other microorganisms through competition for nutrients and production of antimicrobials, such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, and bacteriocins (Aymerich et al., 2000). Bacteriocins are antimicrobial proteins/peptides produced by different microorganisms with antagonistic activity against closely related bacteria (McCormick et al., 1998). Furthermore, bacteriocins are considered natural biopreservatives, as it is assumed that bacteriocins are degraded by the gastrointestinal tract, and most of the LAB are considered GRAS (generally recognized as safe) by the Food and Drug Administration (Silva et al., 2002).

Bacteriocin-producing lactic acid bacteria have been repeatedly shown to inhibit the growth of pathogens in ready-to-eat meat products. Bredholt et al. (1999) found 5 indigenous *Lactobacillus sake* strains that inhibited the growth of *Listeria monocytogenes* in cooked sliced ham without negatively affecting the sensory qualities. However, bacteriocin production is not always required to negatively affect the growth of pathogenic organisms. Mataragas et al. (2003) found inhibition of *L. monocytogenes* below the enumeration limit ($10^2$ cfu $g^{-1}$) in the presence of a bacteriocin produced by LAB. They also found a decrease of 1.5 $\log_{10}$ cfu $g^{-1}$ in *L. monocytogenes* when inoculated with LAB and no bacteriocin. This suggests a combination of factors may be responsible for the antilisterial effects of lactic acid bacteria.

**2. Role in spoilage/shelf life**

Spoilage is preventable to a great extent by using one or more of several available preservation techniques, which act by preventing or inhibiting microbial growth, such as chilling, freezing, drying, curing, packaging, and adding preservatives (Gould, 1996). When one or more of these methods are employed in refrigerated ready-to-eat products, Gram-negative spoilage flora are inhibited, increasing shelf life dramatically (Jeppesen and Huss, 1993). The major cause of spoilage in these products then transfers to the Gram-positive psychrotrophs, such as the lactic acid bacteria.

Lactic acid bacteria have been identified as the major spoilage-causing bacteria in many cooked meat products, due to the atmosphere created by the vacuum

packaging (Björkroth and Korkeala, 1997). These products include, but are not limited to, sliced vacuum-packed deli meats, smoked pork loin, frankfurters, Vienna sausages, ring sausages, and various German-style wurst sausages. Lactic acid bacteria produce lactic acid, ethanol, acetic acid, hydrogen peroxide, and carbon dioxide (Aymerich et al., 2000). These same compounds that create favorable flavors in certain foods eventually increase in concentration, decreasing the sensory acceptability of the contaminated foods. Depending on the type of product, this quality deterioration usually starts 1 to 4 weeks after packaging, and is manifested mainly as formation of "sour" or "cheesy" off odors and/or off flavors (Björkroth et al., 2000). This spoilage can be very costly for manufacturers.

Many factors influence the growth rate and activity of the spoilage activity, including temperature, atmosphere, pH, salt concentration, and product composition. Blixit and Borch (2002) found spoilage rate was related to initial pH, fat content, and L-lactate, regardless of the origin of the meat (pork vs. beef). Dalgaard et al. (2003) identified lactic acid bacteria strains found in cooked and brine shrimp stored under modified atmosphere between 0 and 25°C. The major organism found in 15-25°C was *Enterococcus faecalis*, while *Carnobacterium divergens* and *Lactobacillus curvatus* were the major organisms in 0, 5, and 8°C.

### 3. *Enterococcus faecalis*

The genus *Enterococcus* has been defined by Carr et al. (2002) as "Gram-positive, facultative anaerobic cocci with growth at 10°C and 45°C, growth in broth with 6.5% NaCl, growth at pH 9.6, and reduction of 0.1% Methylene Blue Milk

Medium." The *Enterococci* are homofermenters, producing lactic acid from glucose, and have the ability to break down amino acids for energy (Stiles and Holzapfel, 1997). Despite belonging to the lactic acid bacteria, there has been some controversy over considering them as GRAS (Generally Recognized As Safe) for usage in foods (Giraffa et al., 1997). The enterococci are normal inhabitants of the intestinal tract and female genital tract which have been occasionally associated with urinary tract infection, bacteremia, and bacterial endocarditis when entering the blood stream (Health Canada, 2001). These infections have occurred mainly in immunosuppressed and intensive care patients, and their link to foodborne disease is questionable (Moellering, 1992).

Enterococci colonize raw foods of animal origin (meat and milk) by intestinal or environmental contamination and can even survive and multiply during fermentation (Giraffa, 2002). They can also be found as spoilage contaminants in processed meats, either by surviving cooking processing due to their thermoduric nature (Lawlor, 1999), or by cross-contamination at the final stages of processing, such as slicing and packaging (Hugas et al., 2003). Overall, the persistence of enterococci can be attributed to their wide range of growth temperatures and their high tolerance to salt.

Enterococci are also significant in their productions of bacteriocins. Vignolo et al. (2000) examined three bacteriocins, one from *E. faecium*, for antilisterial ability. Not only were all three bacteriocins effective at inhibiting *Listeria* to a degree, but when used in combination with each other, the antilisterial capability was increased. Marekova et al. (2003) found similar activity in a different strain that produced two

separate bacteriocins with antilisterial ability. These bacteriocins were also shown to have a synergistic effect on each other, having a stronger inhibitory effect on *Listeria* when both were present. Leroy et al. (2003) reviewed a strain of *Enterococcus faecium* for use as a protective co-culture in food fermentation and found strong antilisterial activity. In addition, this activity was enhanced by a boost of bacteriocin activity in very early growth phase, where as most LAB produce bacteriocin in a growth-associated way. These studies suggest many LAB strains have possible applications in food processing and safety.

**4. *Carnobacterium gallinarum***

*Carnobacterium* is a recent genus, reclassified based on DNA homology and physiological studies. (Collins et al., 1987) The genus includes previously named "atypical meat lactic acid bacteria" along with *Lactobacillus divergenes, L. piscicola*, and several other rod-shaped psychrotrophs. *Carnobacterium gallinarum* is a Gram-positive, nonsporeforming, short rod often found in pairs or short chains. It is not motile at 25°C and shows no hemolysis on blood agar (Lawlor, 1999). The organism has the ability to grow at 0°C, but not at 45°C and is inhibited by acetate and pH <6.0 with an optimal pH in the range of 8.0-9.0 (Carr et al., 2002).

In addition to spoilage of meat, most often poultry, *Carnobacterium* species have been isolated from unprocessed chilled beef, lamb, and pork (Carr et al., 2002). Borch et al. (1996) identified *Carnobacterium* as one of the two major spoilage organisms in fresh beef and pork. Jones (2004) found a succession of lactic acid

bacteria species in drip samples from vacuum-packed fresh beef strip loins, with *Carnobacterium* as a top contaminant.

*Carnobacterium spp*. have also been identified as potential bacteriocin producers, and hence, have been investigated for antipathogenic abilities. Duffes et al. (1999) found strains of *C. divergens* and *C. piscicola* with antilisterial ability as early as the fourth day of growth in a model cold-smoked fish product. Silva et al. (2002) used a *Carnobacterium* strain recognized for bacteriocin ability in a new spray drying technique. The strain survived the process and retained antilisterial ability, although it did lose activity against *Staphylococcus aureus*. In a study performed by Nilsson et al. (2004), a *Carnobacterium* strain with bacteriocin and a mutant with no bacteriocin capability were both examined against *Listeria monocytogenes*. While the $bac^+$ strain suppressed *L. monocytogenes* by 6 log, the $bac^-$ strain suppressed *L. monocytogenes* by 3 log, indicating that bacteriocins are not always the only method for pathogen reduction.

**5. *Lactobacillus plantarum***

The genus of *Lactobacillus* is divided based on characteristics of fermentation: (1) obligately homofermentative; (2) facultatively heterofermentative; and (3) obligately heterofermentative. Species from all of these groups are used in fermented foods, but group 3 is most often associated with food spoilage (Stiles and Holzapfel, 1997). *Lactobacillus plantarum* is designated group 2 because it is used as a starter culture in fermented sausages and other food products. *L. plantarum* is historically one of the earliest recognized strains for food fermentation, with patents

applied for the usage in 1940, and introduced as starter organisms in Europe and the USA during the mid-1960's (Hammes et al., 1990).

*Lactobacillus plantarum* is a Gram-positive, nonsporeforming short rod often found in small chains (Lawlor, 1999). It is often found in nature within plant and dairy products, and has been identified as a native strain in both cabbage and cheese. It belongs to a group called *Streptobacteria*, which ferment both lactose and maltose. Unlike atypical *Streptobacteria*, it does not ferment mannitol (Carr et al., 2002). It is a psychrotroph, and can grow at temperatures as low as 2°C, albeit very slowly.

The dominant microbiota in many meat products is comprised of several species of lactic acid bacteria, mainly of the genus *Lactobacillus*, e.g. *L. sakei, L. curvatus,* and *L. plantarum* (Hugas et al., 2003). The spoilage of these products is often indicated by sour spoilage odors and flavor after a period of time under refrigerated storage (Samelis et al., 2000).

Recent studies indicate some strains of *Lactobacillus* are bacteriocin producers, including but not limited to *L. plantarum, L. lactis, L. casei,* and *L. sake* (Vignolo et al., 2000; Hugas et al., 2003; Maldonado et al., 2004). Jeppesen and Huss (1993) found a *L. plantarum* strain with antagonistic effects against *Listeria* in a model fish product containing citric acid or NaCl as curing agents. Much more recently, a strain of *L. plantarum* was studied for its bacteriocin production. It was found that the presence of bacteriocin was induced by the presence of other Gram-positive organisms, such as *Listeria* and other pathogens, or by strains of *L. plantarum* that had been previously exposed to those other Gm(+) organisms (Maldonado et al., 2004).

## D. Chill brines

### 1. General information

Bacterial recontamination of thermally processed meat products is directly linked to food safety and shelf life. Chill brines are used to quickly cool thermally processed food products in order to prevent bacterial growth between 40-140°F. This helps to lengthen the shelf life and safety of the product by decreasing the numbers of spoilage organisms and pathogens able to grow on the product under lower temperatures. A typical brine chiller system is shown in Appendix II.

As the product is rinsed with the chilled brines, heat and nutrients run off back into the original solution. In many cases, bacteria may survive, grow, and spread in these chilling brines. Many of these brines are recycled, which helps keep costs down for processors. The USDA sets limits for maximum usage of chill brines, based on the salinity and temperature of the brine. The regulations for chill brine maintenance are shown below.

**Table 1**: USDA regulations for recycled chill brines (USDA, 2000)

| Duration of Use | Solution maintenance conditions | |
|---|---|---|
| | Minimum salt concentration (%) | Maximum temperature |
| One production shift | None | None |
| Up to 24 hours | 5 | 40°F |
| Up to 1 week | 9 | 28°F |
| Up to 4 weeks | 20 | 10°F |

Being able to control *L. monocytogenes* in these solutions may further extend the life of chill brines, dramatically reducing the cost of water, salt, brine disposal, and time lost when replacing batches of brine, without fear of increased pathogen risk (Ye et al., 2001).

## 2. Survival of *Listeria monocytogenes* in chill brines

Because *Listeria spp.* are a serious threat to ready-to-eat products, there is increased pressure for processors to use HACCP steps to analyze hazards in their processing lines. Chill brines have been frequently targeted as a source of contamination, which has prompted several studies on *Listeria* survival and inhibition in these brines. A study on recirculating injection brine for pork found an increase of 2.34 log cfu/100 ml in *Listeria monocytogenes* after 2.5 hours of recirculation, along with a dramatic increase in a number of spoilage organisms (Greer et al., 2004).

Miller et al. (1997) evaluated growth, injury, and survival potential of several pathogens under brine chiller conditions and containing 0.5% to 20% salt concentration. While other pathogens were significantly injured under such conditions, *Listeria monocytogenes* survived for 30 days at –12°C in 20% NaCl, indicating low temperatures and high salt concentrations are not enough to prevent the survival of this pathogen. Larson et al. (1999) also found *L. monocytogenes* surviving in cheese brines held at 4°C, regardless of pH, salt, nitrogen, or mineral content.

Other studies have focused on a treatment of brine to lower the pathogen count. Eklund et al. (1995) used a smoke treatment to lower counts of *Listeria monocytogenes* in a brined salmon product. Surface bacterial counts were reduced 10 to 25 fold when below 20°C with applied smoke, although no reduction was seen above 22.2°C regardless of smoking. When *L. monocytogenes* was injected into the

flesh, however, no amount of smoking or low temperatures could inhibit the proliferation of the pathogen, only surface counts were affected.

Ye et al. (2001) used an electrochemical treatment system to treat fresh and recycled brines. Inhibition of *Listeria* was seen in both brines, although D-values in recycled brines were longer than in the fresh brines, 2.5 min at 35mA/cm$^3$ versus 1.61 min at 7mA/cm$^3$, respectively. Gailunas (2003) used an ultraviolet (UV) treatment system for the reduction of *Listeria monocytogenes* and lactic acid bacteria in a model brine product, which yielded at least a 4.5 log reduction in both the pathogen and the spoilage organisms. Both of these studies show great promise for an in-line treatment system, making implementation simple and keeping cost low for producers.

## E. Summary

Many recent studies in food safety have investigated non-thermal processing techniques for ready-to-eat food products. The ideal technology would be easy to implement into a processing facility, less costly than thermal treatments, and well received by consumers. Because lactic acid bacteria are used in the production of many foods on the market today, consumers consider them a natural presence in many products. To be able to use LAB to reduce pathogen counts, thereby increasing food safety, would be beneficial to both processors and consumers.

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# Survival of *Listeria monocytogenes*, *Listeria innocua*, and Lactic Acid Bacteria Species in Chill Brines

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## INTRODUCTION

*Listeria monocytogenes* has been implicated in several deadly outbreaks of foodborne listeriosis in the United States and around the world. The USDA and FDA are both currently enforcing a "zero tolerance" policy for *L. monocytogenes* in ready-to-eat foods in the United States. This organism is halotolerant, resistant to freezing temperatures, and can grow and multiply during refrigeration, where other competing organisms may not. Because of the pervasive nature of *L. monocytogenes*, it is difficult for processors to fully eliminate the organism from the environment.

Chilled salt brines are often used to cool thermally processed frankfurters and deli meats. This rapid cooling prevents the growth of many pathogenic organisms that survive the initial heat treatment. Heat and nutrients are transferred to the brines during the chilling process, increasing the risk of bacterial contamination. Because *L. monocytogenes* can survive at high salt concentrations and refrigeration temperatures, a treatment is necessary to prevent food-borne illness from contaminated brines that are recycled through the chiller system.

Various species of lactic acid bacteria (LAB) present during production are the major contributors to spoilage of frankfurters. Both *L. monocytogenes* and lactic acid bacteria can grow and thrive under similar conditions. This competition for resources has the capability to reduce the number of pathogenic organisms. In addition, some LAB produce bacteriocins with antagonistic activity against *L. monocytogenes*. Studies performed on various meat products have shown that inoculation with strains of LAB can inhibit the growth of *L. monocytogenes*, thereby increasing food safety.

MATERIALS AND METHODS

## A. Culture preparation and maintenance

1. *Listeria monocytogenes* and *Listeria innocua*

Six strains of *Listeria monocytogenes* and one strain of *Listeria innocua* were used in this study. *Listeria monocytogenes* Scott A, Brie, V7, D43, LCDC, ATCC 19115, and *Listeria innocua* ATCC 33090 were obtained from the Department of Food Science and Technology (FST), Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, Virginia. The cultures were prepared by inoculating 10 ml of tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (YE; Difco) (TSBYE) with 1 ml of a frozen culture of one strain of *L. monocytogenes*, and incubating at 32°C for 48 hours. This procedure was repeated for each of the six strains used. One loop of this culture was streaked on Modified Oxford agar (Oxford Medium Base plus Modified Antimicrobic Supplement; Difco) (MOX) for isolation and incubated at 32°C for 48 hours. Colonies were confirmed using *Listeria* API strip (BioMérieux, Inc.; Hazelwood, Missouri). Established colonies were transferred back into 10ml of sterile TSBYE and incubated at 32°C for 24 hours. Each strain was then streaked onto slants of tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE). The slants were maintained at 4°C for the remainder of the study. Prior to use, one loopful of culture from each slant was grown in 10ml TSBYE and incubated at 32°C for 24 hours. A cocktail of *Listeria monocytogenes* was made by combining equal proportions of each of the six strains suspended in TSBYE. A cocktail of *Listeria innocua* was made by combining six equal proportions of the same LI strain

suspended in TSBYE. The mixture was then centrifuged at 10,000 x g for 5 minutes and washed with sterile water. The washed cells were centrifuged again at 10,000 x G for 5 minutes and resuspended in sterile water. The cocktail was stored in a sterile container at 4°C for no longer than two hours before use.

2. Lactic Acid Bacteria

Three strains of lactic-acid producing bacteria were used in this study: *Carnobacterium gallinarum* (ATCC 49517) isolated from ice slush from chicken carcasses, *Lactobacillus plantarum* (ATCC 49445) isolated from ground pork, and *Enterococcus faecalis* (ATCC 29212) isolated from humans. All were obtained from the Department of Food Science and Technology (FST), Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, Virginia. *Carnobacterium gallinarum* and *L. plantarum* were chosen to represent typical post-processing contaminants and *E. faecalis* was chosen to represent thermoduric nonsporeformers that can potentially survive thermal processing. *Carnobacterium gallinarum* and *E. faecalis* were maintained at 4°C on slants of All-Purpose Tween agar (APT; Difco). *Lactobacillus plantarum* cultures were maintained at 4°C on slants of deMan, Rogosa, and Sharpe agar (MRS; Remel Inc., Lenexa, KS). Prior to use, one loopful of each strain was grown in 10ml APT broth (for *C. gallinarum* and *E. faecalis*) or MRS broth (for *L. plantarum*) and incubated at 30°C for 24 hours. A cocktail of lactic acid bacteria (LAB) was made by combining two equal proportions of each of the three strains suspended in APT or MRS. The mixture was then centrifuged at 10,000 x G for 5 minutes and washed with sterile water. The washed cells were

centrifuged again at 10,000 x G for 5 minutes and resuspended in sterile water. The cocktail was stored in a sterile container at 4°C for no longer than two hours before use.

3. Mixed inoculum

In cases where a cocktail contained both *Listeria* (either *monocytogenes* or *innocua*) and lactic acid bacteria, the cocktails were made separately according to the previously stated method. After centrifugation, equal portions of the two cocktails were combined to give a cocktail with 50% *Listeria* and 50% lactic acid bacteria. The cocktails were then stored at 4°C for no longer than two hours before use.

**B. Brine preparation**

The brine solutions were prepared in the laboratory facilities 24 hours before inoculation. All equipment used for brine preparation were cleaned using hot water and Sparkleen laboratory detergent (Fischer Scientific; Pittsburg, PA). The brines were prepared by dissolving Cargill Top-Flo Evaporated Salt (99.8% purity) (Cargill Inc., Minneapolis, MN) into domestic tap water until a 7.9% w/v or a 13.2% w/v solution was attained.

The 7.9% w/v brine was prepared by dissolving 79g of salt into 1 L of tap water in a glass container. The 13.2% w/v brine was prepared by dissolving 132g of salt into 1 L of tap water. The solution was mixed on a stir plate until dissolved, pipetted into individual 10 ml screw-cap tubes, and then autoclaved to ensure sterility. The brines were stored in a 4°C or 12°C incubator for 24 hours before use.

Top-Flo Evaporated Salt has been approved by the U.S. Department of Agriculture Food Safety and Inspection Service for direct use in meat and poultry products. The salt is of food grade quality, in compliance with the standards for sodium chloride set forth in the Food Chemicals Codex. Water-soluble yellow prussiate of soda has been added to the salt to improve caking resistance in conformity with 21CFR 172.940. A chemical analysis of the salt can be found in Appendix 1.

**C. Inoculation, incubation, and sampling**

In screw-cap tubes, 10 ml of tap water, 7.9%w/v brine, or 13.2%w/v brine were inoculated with one of the following: *Listeria monocytogenes, Listeria innocua*, lactic acid bacteria, *L. monocytogenes* + lactic acid bacteria, or *L. innocua* + lactic acid bacteria, yielding 15 brine and inoculum combinations. Each screw-cap tube was inoculated with 200 µl of cocktail to yield an initial count of ~6 $\log_{10}$ CFU/ml and incubated at 4°C or 12°C. Each tube was considered representative of one time period and discarded after sampling in order to avoid contamination that may occur when sampling frequently from a larger container. At the sample time (0, 2, 4, 6, 8, and 10 days), an unopened tube was serially diluted with 0.1% peptone (Difco) water as necessary and pour plated in duplicate. Each brine and cocktail combination was analyzed in triplicate.

**D. Microbial analysis**

1. *Listeria monocytogenes* and *Listeria innocua*

Samples containing *L. monocytogenes* or *L. innocua* were pour plated in duplicate on Modified Oxford agar (MOX) (Oxford Medium Base plus Modified Antimicrobic Supplement; Difco) and incubated at 32°C for 48 hours. After incubation, the esculin-positive (black) colonies were counted and recorded as CFU/ml brine. Initial trials were also plated on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE) to rule out the possibility of injured cells that may not thrive on MOX. Upon confirming comparable results on both MOX and TSAYE, the TSAYE plating was discontinued.

2. Lactic Acid Bacteria

Samples containing lactic acid bacteria were pour plated in duplicate on All-Purpose Tween (APT) agar (Difco) containing 0.0032% (w/v) bromocresol purple (BCP) (Fisher Scientific, Pittsburgh, PA) (APT+BCP) as an acid indicator and incubated at 30°C for 48 hours. After incubation, the sub-surface acid-producing (yellow) colonies on APT+BCP (purple background) were counted and recorded as CFU/ml brine.

3. *Listeria* species + Lactic Acid Bacteria

Samples containing one of the *Listeria* species + lactic acid bacteria were pour plated in duplicate on Modified Oxford agar (MOX) to enumerate only the *Listeria* species present. After incubation, the esculin-positive (black) colonies were counted and recorded as CFU/ml brine. The plates were also pour plated in duplicate on All-Purpose Tween (APT) agar containing 0.0032% bromocresol purple (BCP) as an acid

indicator to enumerate only the lactic acid bacteria species present. After incubation, the sub-surface acid-producing (yellow) colonies on APT+BCP (purple background) were counted and recorded as CFU/ml brine. Initial trials were also plated on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE) to determine if the counts from MOX and APT+BCP selective agar were representative of bacterial populations plated on non-selective agar. Upon confirmation, the TSAYE plating was discontinued.

## E. Statistical analysis

Treatments were arranged as a 5 x 3 x 2 factorial in a randomized complete block design. Each plate count was performed in duplicate and the entire study was repeated three times. Bacterial counts were transformed into base-10 logarithms. Analysis of variance was used to determine if significant differences ($p<0.05$) existed between microbial populations in different brine concentrations (7.9% and 13.2% w/v), at different temperatures (4°C and 12°C), with or without the presence of competitive lactic acid bacteria. The different combinations of variables were analyzed separately using JMP statistical software (SAS Institute Inc., Cary, N. C.). Means were separated using the least significant difference method with a significance level of 0.05.

# RESULTS AND DISCUSSION

The presence of *Carnobacterium gallinarum, Enterococcus faecalis,* and *Lactobacillus plantarum* were not effective at consistently reducing the population of *Listeria spp.* across all brine concentrations. The initial *L. monocytogenes* inoculum concentrations averaged 7.1 $\log_{10}$ cfu/ml over all brine concentrations and temperatures. Populations of *L. monocytogenes* alone were not significantly reduced at any salinity in the control groups. This is consistent with the work of Bal'a and Marshall (1996), who found *L. monocytogenes* had an increased ability to tolerate higher salt concentrations at lower incubation temperatures (5-15°C). As seen in Figure 1, *L. monocytogenes* was significantly reduced by lactic acid bacteria by an average of 1.4 log at 4°C and 3.0 log at 12°C in 0% salinity, or water ($p<0.05$). In the 7.9 and 13.2% salinities however, no significant reduction was observed at either temperature, which is shown in Figure 2. The ineffectiveness of LAB at reducing *L. monocytogenes* populations in the high salinity brines may possibly be due to the unique salt-stress mechanism of *L. monocytogenes*. Duché et al. (2002) found that *L. monocytogenes* produces 12 salt-shock proteins under osmotic stress (3.5% w/v NaCl). These proteins protect *L. monocytogenes* against rapidly changing environments. In this case, *L. monocytogenes* was more susceptible to the competition of LAB in the 0% salinity, but when salt-shock proteins were produced in 7.9 and 13.2% salinity, the pathogen was much less susceptible to the inhibitory effects of LAB. This is further reinforced by Vialette et al. (2003), who observed *L. monocytogenes* was less susceptible to acid stress when in higher concentrations of salt. Because one of the main inhibitory effects of LAB is acid production, it may be possible

that LAB is ineffective at reducing populations of *L. monocytogenes* in high-salinity environments.

*Listeria innocua* inoculum concentrations averaged 7.49 $\log_{10}$ cfu/ml over all brine concentrations and temperatures. As seen in Figure 3, *Listeria innocua* population was not significantly reduced in the presence of lactic acid bacteria at either 4 or 12°C. In fact, populations of *L. innocua* with LAB were significantly higher by day 10 than the control group with no LAB ($p<0.05$). A possible explanation for this effect was explored by Kalmokoff et al. (2001), who identified a bacteriocin produced by *L. innocua* strain 743 that acted much like a LAB pediocin. If our strain of *L. innocua* had an antagonistic effect on LAB, it might survive better in the brines containing LAB. This is unlikely, however, as our strain of *L. innocua* has been clearly identified as ATCC 33090 and has never been shown to produce a bacteriocin of any kind. A more likely scenario would be to attribute this effect to the salt and acid resistance of *Listeria* species demonstrated by Vialette et al. (2003), who found a synergistic effect between acid stress and salt stress in *L. monocytogenes*. If this effect held true for *L. innocua*, the populations subjected to acid production by LAB would be able to better survive the high salinity environment than the *L. innocua* control with no LAB. Because LAB acid production lowers the pH slowly over time, this effect would not be seen immediately, but would emerge over time, as in this case of a 10-day storage. This effect is most likely responsible for the increased survival of *L. innocua* in the presence of LAB.

The initial concentration of lactic acid bacteria averaged 7.0 $\log_{10}$ cfu/ml over all brine concentrations and temperatures. These concentrations remained relatively constant, decreasing only slightly (< 1 log) over the course of each trial. Lactic acid

bacteria counts were significantly reduced ($p<0.05$) in 0% salinity in the *L. monocytogenes* treatment groups (1.2 log reduction in 4°C, 2.3 log reduction in 12°C), but not in the 7.9 or 13.2% salinity brines. No other significant differences occurred in LAB populations in the presence of *Listeria spp.* when compared to the control. This reduction was probably due in part to the lack of nutrients available in the sterilized water. *Listeria* is considered an environmental contaminant due to its remarkable survival abilities in a variety of harsh environments, but LAB may require more available nutrients for survival. The LAB could survive in the water when alone or with the less competitive *L. innocua*, but when challenged by the presence of *L. monocytogenes*, the population began to slightly decline. This is in contrast with Samelis et al. (2001), who observed a small decline in pathogens stored in water washings that had been filter-sterilized, which was concluded to be due to lack of available nutrients. The natural flora was able to survive in the sterile water washings, however, that population was determined to be mostly *Pseudomonas*, and not necessarily the LAB that were used in this instance.

In general, LAB was not effective in the reduction of either *Listeria* species. The acidic pH effects that LAB produce were apparently overcome by *Listeria* species through the acid/salt-tolerance mechanisms explained previously. Some LAB produce bacteriocins, which have been shown by several studies to be effective against *Listeria* (Nilsson et al., 2004; Vignolo et al., 2000; Bredholt et al., 1999). Apparently in this case, the LAB used did not produce an effective bacteriocin against *Listeria*. Nilsson et al. (2004), found that a bacteriocin negative mutant of *Carnobacterium* had a significant nonbacteriocin-dependent inhibitory effect on *Listeria*. This is contrary to our findings,

which indicates the importance of the specific LAB strains used on the outcome of any study. Our results concurred with Duffes et al. (1999), who tested 100 LAB strains for bacteriocin activity against *L. innocua*, finding that only 22 had inhibitory ability. Because the LAB in this study failed to decrease or inhibit populations of *Listeria*, it can be inferred that they are in the group of bacteriocin-negative LAB.

*Listeria innocua* was used in addition to *L. monocytogenes* in order to test *L. innocua* for its ability to model for *L. monocytogenes* under these conditions. As shown in Figure 4, these two organisms were significantly different in their survival in the control brines with no LAB ($p<0.05$). *Listeria innocua* was more susceptible to the effects of the brines and LAB than the pathogenic *L. monocytogenes*. These statistical differences began on day 2 in some cases, and by day 4 in all salinities. This difference between the two *Listeria* species was also seen when LAB was present in the 7.9% and 13.2% NaCl brines, as seen in Figure 5. No significant differences were seen between *Listeria* species in the 0% NaCl brine. This concurs with the observations made by Meylheuc et al. (2002), who investigated growth characteristics and cell surface properties of *L. innocua* and *L. monocytogenes*. Marked differences were found in the physiochemical makeup of the two species, specifically among cell membrane function of *L. innocua*, which was found to be more electronegative and more variable in its Lewis acid-base characteristics as a function of temperature. Membrane function is essential in the mechanism for surviving environmental stressors, such as salt, pH, and water activity (Russel et al., 1995). If *L. innocua* differs from *L. monocytogenes* in membrane functions, it is conceivable that the survival abilities of *L. innocua* would differ as well. Meylheuc et al. (2002) concluded that these membrane functions contributed greatly to

the pathogenicity of *L. monocytogenes*; the non-pathogen *L. innocua* is less virulent in part because of these differences. In this experiment, it is apparent that *L. innocua* is not an acceptable model for the behavior of *L. monocytogenes* under these environmental conditions.

Trials performed at 4°C and 12°C were found statistically different overall ($p<0.05$). The lower temperature had a protective effect on the survival of both *Listeria* species in the brines, whereas a greater inhibition of both species occurred at 12°C. At lower temperatures, an effect known as homeoviscous adaptation occurs. By changing the lipid composition of their membranes, many organisms can compensate for temperature changes. Increasing the degree of unsaturation in these membrane lipids decreases their melting point, allowing cell membranes to maintain a fluid nature and thus allows membrane proteins to function (Hazel, 1995). A second cell mechanism for survival at lower temperatures is the production of cold shock proteins (CSPs), which allow for increased environmental adaptability. *Listeria monocytogenes* has been found to have 12 CSPs that work to stabilize the cells when exposed to low temperatures (Bayles et al., 1996). These CSPs are found in nearly all bacteria and act to minimize secondary folding of cellular components, which is one mechanism of decreased cell activity and eventual death (Wemekamp-Kamphuis et al., 2002). Production of cold shock proteins in this instance increased cell survival in the 4°C group, regardless of other treatments employed.

Because sodium chloride is chemically neutral and possesses no buffering action, the pH of these NaCl brines depends solely on the quality of the water used (Gailunas, 2003). Brines made at processing facilities may vary in pH depending on what acidic or

alkaline materials might be present as contaminants in the water. Predictably, the pH of the tap water brines used in this study averaged 7.1 before inoculation, regardless of the salinity. Brines without LAB remained at 7.1 after the day 10 storage period. In the brines innoculated with LAB cocktail, the pH declined to an average of 5.7 after the 10 day storage period. This pH is still well within the lower growth limit of 4.1 examined by Jay (2000). As evidenced by our results, the pH of these brines was likely not an inhibitory factor for *Listeria*.

# CONCLUSIONS

Overall, the strains of LAB used in this experiment were not effective at limiting or inhibiting *L. monocytogenes* in chill brines. Results and treatments using LAB suggest that these organisms are not a universal panacea for every food safety issue. However, the use of LAB shows extraordinary promise in partial inhibitions of certain pathogens under specific environmental conditions and product composition. Because of the amount of literature that suggests LAB can be utilized to inhibit *Listeria*, further studies should be performed using different strains of LAB. These strains may also be made more effective by manipulating environmental conditions to create greater stress on the pathogens. Results of *Listeria* inhibition by LAB also differ based on the composition of the food product being studied. Although this study did not find an effective LAB treatment, there is far too much unknown about the synergistic mechanisms of microbial competition, cell stress, survival, and death to completely abandon the idea of proactively using LAB to inhibit or erradicate dangerous pathogens.