CONTROL OF *LISTERIA MONOCYTOGENES* ON FRANKFURTERS FORMULATED WITH AND WITHOUT LACTATE BY DIPPING IN SODIUM LACTATE AND ACIDIFIED CALCIUM SULFATE BEFORE AND AFTER INOCULATION FOR SHELF LIFE EXTENSION

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ABSTRACT

The objectives of these studies were to determine the antimicrobial effects of sodium lactate (SL) and acidified calcium sulfate (ACS) on frankfurters formulated with and without lactate in the frankfurter formulation. Two studies were performed, one which mimicked home storage, and the other evaluated the effectiveness of SL (12% v/v) and ACS (12% v/v) as antimicrobial dips when used prior to and after inoculation of *Listeria monocytogenes* on frankfurters formulated without lactate. In the first study, five peeled frankfurters with and without lactate in the formulation were either dipped in SL or in ACS, stabilized for 30 minutes, vacuum packaged and stored for 30 days at 4°C. Controls were also prepared by dipping in 0.1% peptone. After 30 days the packages were opened and frankfurters were dip inoculated, stabilized for 30 minutes, and one frankfurter from each treatment was sampled. All other frankfurters were then placed in storage at 7°C and sampled after an additional 7, 14, and 21 days. For the second study, treatments consisted of five frankfurters that were first inoculated with a five-strain cocktail of *L. monocytogenes*, stabilized for 30 minutes, then dipped in SL or acidified ACS; or were first dipped in SL or ACS, stabilized for 30 minutes then dip inoculated. Controls were prepared by dip inoculating frankfurters. One frankfurter from each treatment was sampled immediately. The remaining frankfurters were vacuum packaged, stored at 4°C and sampled after 30, 60, 90, and 120 days. For both studies, on sampling days one frankfurter from each treatment was pulsified and plated on Tryptic Soy Agar (TSA) for viable cell counts and Modified Oxford Medium (MOX) for *L. monocytogenes* counts. The results indicated that SL dipped frankfurters had lower total aerobic counts and *L. monocytogenes* counts compared with ACS treatments and the controls. Use of lactate formulation in frankfurters resulted in lower bacterial counts of both natural microflora and inoculated *L. monocytogenes* in frankfurters after prolonged storage at 4 °C. This research indicates that sodium lactate (12% v/v) may be effective as an antimicrobial dip on frankfurters for the reduction of natural microflora and *L. monocytogenes*.

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PREFACE

The Centers for Disease Control and Prevention estimate that 2500 illness and 500 deaths occur each year due to listeriosis (CDC, 2005). Unlike most bacteria, *Listeria monocytogenes* is able to grow at 4°C in vacuum packages (Glass and Doyle, 1989). Of special concern is Ready-to-Eat (RTE) meat and poultry products as these items can become contaminated post-processing and may be eaten with little or no heating by the consumer. In addition, in food production practices such as increased centralization of food production facilities and a growing at-risk population (children, the elderly, AIDS and cancer patients and other immunocompromised individuals) will likely make listeriosis a continued health threat. Due to this concern, US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) and the Food and Drug Administration (FDA) have a "zero tolerance" policy for *L. monocytogenes* in RTE products. In addition to this, USDA-FSIS has required that federally inspected establishments that produce ready-to-eat (RTE) meat and poultry products use one of the following alternatives for the control of *Listeria monocytogenes* (FSIS Directive 10240.4, 2009).

I. Use of a post-lethality treatment (which may also be the antimicrobial agent or process) that reduces or eliminates microorganisms on the product AND an antimicrobial agent or process that suppress or limits the growth of *L. monocytogenes.*

II. Use of either post-lethality treatment (which may be an antimicrobial agent or process) that reduces or eliminates microorganisms on the product OR an

antimicrobial agent or process that suppress or limits the growth of *L. monocytogenes*.

III. Use of sanitation measures only.

Despite the advancements made in food safety completely eliminating *Listeria monocytogenes* from the food supply remains a challenge for food manufacturers. The FDA is currently reviewing a petition from the food industry to change the zero-tolerance policy for *L. monocytogenes* in foods that do not support the outgrowth of the bacterium. This petition requests that food items have a regulatory limit of 100 CFU/g in foods that do not support the outgrowth of this organism. Arguing that concentrating on the number of bacteria present rather than presence alone may be more effective in improving food safety.

As a response to increased consumer awareness and heightening regulations on the food industry for the control of *L. monocytogenes*, companies will likely increase the use of additives with antimicrobial, bactericidal, or bacteriostatic properties in the product formulation and/or applications to product surfaces. In recent years the Department of Health and Human Services launched the "Healthy People 2010" initiative. One of the 28 main focus areas of this program is to improve food safety in the United States, including reduction in the incidence of foodborne diseases caused by *L. monocytogenes, Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* (http://www.healthypeople.gov/About/goals.htm). This initiative will cause additional interest in the food industry to improve food safety and quality by the use of food additives. Further incentives for the food industry to provide safer food is consumer

perception of food companies' responsibility to provide safe food. In a survey conducted by Cates et al. (2006) more than 75% of those surveyed believed that food manufacturers have a lot of control over ensuring the safety of the U.S. food supply.

Further complicating this matter is the present demand by consumers for reduced amounts of salt and preservatives in products, along with longer shelf life, smaller packages and greater convenience.

LITERATURE REVIEW

Genus *Listeria* and *Listeria monocytogenes*

For many years after its discovery the genus *Listeria* only contained the *Listeria monocytogenes* species. In 1948 *L. denitrificans* was added because of its ability to reduce nitrates, *L. grayi* added in 1966 (in honor of M.L. Gray, an American microbiologist), *L. murrai* added in 1971 (in honor of E.G.D. Murray, a Canadian microbiologist), *L. innocua* added in 1981 (named because of its harmlessness), in 1985 *L. ivanovii* was added (in honor of I. Ivanov, a Bulgarian microbiologist), in 1983 *L. welshmeri* (in honor of H.J. Welshimer, an American microbiologist), and finally in 1983 *L. seeligeri* (in honor of H.P.R. Seeliger, a German microbiologist) was added. The genus *Listeria* currently includes six species; *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi, L. ivanovii* and *L. monocytogenes*. Of these species only *L. monocytogenes* is generally considered to be capable of causing human illness. However, Snapir et al. (2006) reported a case of listeriosis due to *L. ivanovii*. These authors suggest that *L. ivanovii* may cause human illness in extremely rare cases, finding only six cases of infection documented.

In 1924 E.G.D. Murray isolated a bacterium from the blood of laboratory animals, specifically rabbits, and could not assign it to any known genus. He therefore named the newly discovered bacterium *Bacterium monocytogenes* (Murray et al., 1926). He derived the name for the new bacterium from the large mononeuclear leucocytosis that it caused in the rabbits. In 1940 the bacterium was renamed *Listeria* for catalase-positive, Gram-positive rods (Pirie, J.H.H. 1940) in honor of British surgeon Lord Joseph Lister. *L.*

monocytogenes has also been named *L. bovina*, *L. cunniculi*, *L. gallinarum*, *L. gerbilli*, and *L. suis* and many others (Cliver, 1990).

Morphology

Listeria monocytogenes is a short, small (1.0-2.0 µm in length x 0.5 µm in diameter), regular Gram-positive, non-spore forming rod with rounded ends. Cells may be found as single units or in short chains, they may also be arranged in V or Y forms. The cells sometimes are coccoid, averaging about 0. 5 µm in diameter, and may be confused with streptococci. Old cultures can lose the ability to retain Gram stain and may be mistaken for *Hemophilus*.

Culture

Listeria monocytogenes is non-acid fast, can be aerobic, microaerophilic or facultatlively anaerobic; and can grow at temperatures between 1 - 45°C (Saunders and Wiedmann, 2007), with an optimum growth temperature being between 30 - 37°C. The bacterium is Voges-Proskauer positive, can produce β-hemolysis on blood agar, and is catalase positive. *Listeria monocytogenes* can grow at a wide range of pH from 4.3 to 9.6 and can survive high salt concentrations (up to 10%). *Listeria* is one of the few pathogens that can grow at a_w 0.93 (Petran, 1989). Growth factors for this bacterium include; cystine, leucine, isoleucine, arginine, methionine, valine, cysteine, riboflavin, biotin, thiamine, and thioctic acid (Premaratne et al., 1991, Siddiqi and Khan, 1989, Welshimer, 1963).

Glucose and glutamine are required as primary sources of carbon and nitrogen (Premaratne et al., 1991). This bacterium is motile at room temperature via peritrichous flagella that give it its tumbling motility. These abilities allow *L. monocytogenes* to not

only survive but to thrive and multiply under these types of conditions which are commonly used for food preservation which makes this pathogen especially problematic to the food industry. This is also the reason that *L. monocytogenes* is a common food contaminant and a major cause of food recalls due to bacterial contamination in the developed world.

Listeria in the Natural Environment

Because of *Listeria monocytogenes* importance as an animal and human foodborne and feedborne pathogen it is important to gain an understanding distribution and ecology in the natural environment. *L. monocytogenes* is generally considered ubiquitous in nature and has been specifically isolated from soil (Weis, 1975), water (Watkins and Sleath, 1981), vegetation (Weis and Seeliger, 1975; Welshimer, 1981), sewage (al-Ghazali and al-Azawi, 1988a, al-Ghazali and al-Azaw, 1988b, Colburn et al., 1990, and Frances et al., 1991), animal feeds (Caro et al., 1990, Fenlon, 1985, Fenlon, 1986, Ryser et al., 1997, Skovgaad and Morgen,1988), farm environments (Fenlon et al., 1996, Ueno et al., 1996, and Vlaemynck, 1994) and food processing environments (Destro et al., 1996, Harvey and Gilmour, 1994, Hoffman et al., 2003, Jacquet et al., 1993, Lawrence and Gilmour, 1994, Pritchard et al., 1995, and Rorvik et al., 1995). This bacterium has also been isolated from dust, water, healthy human and animal carriers (including 42 domestic and wild mammalian species and 22 avian species), and silage (Welshimer 1981.; Gray et al., 2006; Cliver, 1990). *Listeria monocytogenes* and *Listeria innocua* are the most common *Listeria* spp. found in the natural environment.

Among domestic animals cattle, sheep and goats are most often afflicted by listeriosis (Dutta and Malik, 1981). In ruminants the most commonly presented

syndrome is encephalitis. Infected animals may excrete the bacterium in milk, blood and feces. It may also be possible for asymptomatic cows and goats to excrete high levels of *L. monocytogenes* in milk (Donnelly, 2006).

L. monocytogenes is capable of invading and replicating in numerous animal cell types. It does this by inducing its own uptake into non-phagocytic cells (Ramaswamy et al., 2007). The mode of infection of *Listeria* consists of: adhesion to, and invasion of host cells, escape from phagocytic vacuoles, actin-based intracellular movement and cell-to-cell spread (Pust et al., 2005).

Mechanisms of Adaptation

Survival of *Listeria monocytogenes* at low temperatures, under osmotic stress, and under acid stress involves many mechanisms such as; changes to the composition of the cell membrane, changes in gene expression and induction of proteins, and accumulating cryoprotectants and osmoprotectants.

Bacterial cell membranes are in a fluid, liquid-crystalline state. It is essential for the survival of the bacteria to remain in this physical state which is required for proper structure and function of the membrane. Bacteria maintain this fluid crystalline state in their membranes at low temperature by altering their fatty acid composition. The fatty acid profile of *L. monocytogenes* is characterized by a high proportion of iso and anteiso, odd number fatty acid chains, specifically; $C_{15:0Br}$, $C_{14:0}$, $C_{16:0}$ and , $C_{17:0Br}$ (Raines et al., 1968). This fatty acid profile is what allows this bacterium to survive and multiply at low temperatures. One of the major changes to the fatty acid composition of the membrane at

low temperatures (7°C) is an increase in the proportion of $C_{15:0}$ at the expense of $C_{17:0}$ (Russell, 1992) as well as a slight increase in $C_{18:1}$. An increase in the degree of unsaturated fatty acids is also seen at low temperatures, this helps enhance membrane fluidity. The shortening of fatty acid chain length decreases the carbon-carbon interaction between neighboring chains thus, helping to maintain the optimal membrane fluidity (Russell et al., 1995).

L. monocytogenes posses molecular adaptation capacities that allow them to survive when faced with food associated environmental challenges. This adaptivity allows for resistance to some foodborne preservation techniques including refrigeration (Tasara and Stephen, 2006). These researchers also suggest that cold stress and other food-environmental stresses may actually be selecting for survival and proliferation during processing and storage of foods. *L. monocytogenes* produces cold shock proteins (Csps) and cold acclimation proteins (Caps) in response to temperature downshock, from 37°C to 7°C, and cold acclimation respectively, in cultures grown at 5°C (Bayles et al., 1996). The authors found that 12 Csps were induced when *L. monocytogenes* was subjected to downshock and four Caps were additionally found in the cold acclimated cultures. Bacteria use Csps and Caps to regulate the adaption to cold stress. It is believed that upon cold shock the following occurs: The downshift in temperature is sensed by the cell and signaled to the transcriptional and translational apparatus and possibly to structural elements. These changes are what allow *L.* monocytogenes to not only survive at refrigeration temperatures by to thrive. The Csps are induced immediately after the cold shock, and the production of non-cold shock proteins is transiently inhibited. During this accumulation period the level of Csps is high. After the

accumulation period Csps levels decrease and remain constant and the cell restores protein production of non-Csps (Horn et al., 2007).

The ability of *L. monocytogenes* to uptake and accumulate compounds for the use of osmoprotectant and cyroprotectant provides the bacteria with an increased survivability in adverse environmental conditions. These osmolytes act in the cytosol and counterbalance the external osmolarity and by doing so they prevent water loss from the cell without adversely affecting macromolecular structure and function. In a study done by Bayles and Wilkinson (2000) in which *L. monocytogenes* was grown in high osmotic medium (0.7 M NaCl) and at low temperature (5°C), glycine betaine, proline betaine, acetylcarnitine, carnitine, gbutyrobetaine and 3-dimethylsulphoniopropionate were all found to act as osmoprotectants and cryoprotectants. This led the authors to conclude that the presence of these compounds in foods may provide *L. monocytogenes* a way to overcome the barriers of high osmotic strength and low temperature that otherwise control microbial growth.

Osmotic stress response by microorganisms involves variation of gene expression patterns and physiological changes. Using 2-D gel electrophoresis, Duche et al. (2002), studied the pattern of proteins expressed in salt stressed *L. monocytogenes*. These researchers identified two types of proteins expressed under these conditions; those which were rapidly over-expressed for a short period, and those which were induced rapidly and were continually over-expressed for several hours after return to normal. The proteins were coined as salt shock proteins (Ssp) and stress acclimated proteins (Sap), respectively. Cheroutre-Vialette et al. (1998) found that subjecting *L. monocytogenes* to

osmotic shock increased both the generation time and lag time. The researchers attributed this to the decrease in water activity (a_w). This research shows that while growth of *L. monocytogenes* is hampered by osmotic stress it is not prevented.

When *L. monocytogenes* is exposed to mild acidic conditions (pH 5.5) an acid tolerance response (ATR) is induced. This ATR then allows for cells to become resistant to severe acidic conditions such as pH 3.5 (O'Driscoll et al., 1996). Work done by Phan-Thanh et al. (2000) demonstrated that acid-adapted *L. monocytogenes* (pH 5.2) were more resistant to heat shock (52°C), osmotic shock (25-30% NaCl), alcohol stress, and crystal violet stress (O'Driscoll et al., 1996); indicating that acid adaptation may provide cross-resistance against other environmental stresses. This phenomenon was also seen by Falerio et al. (2003). These researchers further found that lowering the growth temperature to 8°C, did not affect pH sensitivity. The data provided from these studies suggests that ATR may be of special concern to food manufacturers because low-pH conditions may have the potential to select for *L. monocytogenes* mutants with increased natural acid tolerance and increased virulence.

The ability of bacteria to survive harsh environmental conditions may be attributed to changes in the transcription of genes due to an association of alternative sigma factors with the core RNA polymerase. Alternative stress factor, sigmaB has been identified in *L. monocytogenes*. Sigma factor B is stimulated in response to temperature downshift, exposure to an acidic environment, and to high osmotic pressure. Becker et al. (1998) found that stress factor sigmaB to be important for the utilization of both betaine and carnitine as osmoprotectants. Furthermore, Kazmierczak et al. (2003)

showed that virulence gene expression is also regulated by stress factor sigmaB. While it appears sigmaB is an important regulator of stress response of *L. monocytogenes* to environmental stresses, it is important to note that dependence upon sigmaB as a stress response varies among serotypes (Moorhead and Dykes, 2003).

Virulence and Pathogenesis

Listeria monocytogenes has been shown to have a multifactorial virulence system; thiol-activated hemolysin, listeriolysin O, have been identified as playing a crucial role in the organism's ability to multiply within host phagocytic cells and to spread from cell to cell (Farber and Peterkin, 1991). Pathogenesis of human listeriosis infections consist of the following steps: 1) survival of the bacteria in the gastric passage; 2) colonization in the intestinal tract, possibly associated with intestinal symptoms; 3) invasion of epithelial cells; and 4) infection of macrophages followed by systemic spread, septicemia, and infection of the fetus in pregnant women. In cellular infection models the following five steps have been identified: 1) internalization in host cell; 2) escape from the host vacuole; 3) multiplication in the host cell's cytoplasm and movement through the cytoplasm by actin-based motility from proteins of the host cell; 4) movement of the bacteria to the cell surface of the host and formation of protrusions; and 5) neighboring cells then take up these protrusions, followed by escape of the bacterium from the resulting double-membrane vacuole, at which point the cycle repeats. O'Driscoll et al. (1996) suggested that environmental stresses on *L. monocytogenes* during the digestive process may contribute to its infectious life cycle. Furthermore, they found that acid tolerant *L. monocytogenes* was more virulent in mice than non-acid tolerant strains. It has also been shown that the virulence of *Listeria* increases when grown at refrigeration

temperature (rather than optimum) (Durst, 1975; Wood and Woodbine, 1979). Therefore, cold storage may enhance the virulence of some *L. monocytogenes* strains.

There are several factors that play a specific role during infection of host animal with *L. monocytogenes.* These factors can be grouped into two categories: 1) virulence genes, those that are necessary for pathogenesis; and virulence-related genes, which are not essential for pathogenesis but may enhance it.

Koch's postulates state: 1) The suspected causal organism must be constantly associated with the disease. 2) The suspected causal organism must be isolated from an infected plant or animal and grown in pure culture. 3) When a healthy susceptible host is inoculated with the pathogen from pure culture, symptoms of the original disease must develop. 4) The same pathogen must be re-isolated from plants or animals infected under experimental conditions. Virulence genes can be defined by adopting these postulates so that they would require that virulence genes to fulfill the following: 1) the gene must be present in pathogenic strains and absent from, or not expressed, by non-pathogenic strains; 2) disruption of the gene should reduce pathogenesis; and 3) the gene is expressed when the pathogen is in the host environment. Many genes possibly contribute to the pathogenesis and virulence of *L. monocytogenes*. Portnoy et al. (1992) describes hemolysin, listeriolysin O (LLO) as the best characterized factor of pathogenesis. Listeriolysin O is believed to initiate lysis of bacterium-containing vacuoles allowing for intracellular pathogenesis. LLO binds to cell membranes and creates pores. LLO is a thiol-activated toxin similar to streptolysin O, it is active only on cholesterol-containing membranes at low pHs. Production of lechithinase is thought to also be involved in lysis

of the double-membrane vacuole that is formed during intercellular spread (Vazquez-Boland et al., 1992). The gene actA, which codes for a surface protein, has been shown to be necessary for actin assembly which allows *Listeria* intracellular and intercellular movement (Gray et al., 2006). Portnoy et al. (1992) showed that the presence of internalin A (*inlA)* and internalin B (*inlB*) to a lesser degree, are genes that allow for *Listeria* to invade epithelial cells by interaction with E-cadherin. Gene products such as p60 and SvpA, have been identified as surface proteins that are implicated in adherence to and invasion of the host cell. Virulence-related genes that encode protein, such as RecQ and Dfp play a role in DNA repair and replication. The genes *inlC* and *inlD* are considered to be virulence related even though the function of these genes is not known. This is because they are homologs to known virulence genes (ILSI, 2005).

Listeriosis

There is some debate as to when the first actual case of listeriosis in man occurred. According to Cliver (1990), the first confirmed report of listeriosis in humans occurred in 1929 when *Listeria* was isolated from three people who were presenting mononucleosis-like symptoms. However, Ryser and Marth (2007) state that the first confirmed diagnosis was in 1924 in a soldier who was suffering from meningitis at the end of World War II. Of note, a historian has suggested that *L. monocytogenes* could be the root cause of Queen Anne's 17 miscarriages.

There is strong evidence that the majority of human listeriosis cases is from foodborne infections, usually from contaminated ready to eat food (Genigeorgis et al.,

1989). Listeriosis is the infection associated with the consumption of infected foods with the bacterium *Listeria monocytogenes*, it is clinically defined when the organism is isolated from blood, umbilical cord blood, lochia, tissue obtained by curettage, urine, placental tissue, cerebrospinal fluid or any otherwise normally sterile site.

There are two primary syndromes associated with listeriosis, as an invasive and a noninvasive form (ILSI, 2005). The incubation period ranges from several hours for the noninvasive form to several days to weeks for the invasive form, thus, making it difficult to identify the causative source. The invasive form of listeriosis is characterized by the onset of meningitis, septicemia, primary bacteremia, endocarditis, nonmeningitic central nervous system infection, conjunctivitis, confusion, loss of balance, a stiff neck, convulsions and flu-like symptoms (such as fever, headache, fatigue abdominal pain, and malaise). In the United State *Listeria* induced meningitis is the fifth most common bacterial meningitis. The noninvasive form results in febrile gastroenteritis. The susceptible population for listeriosis has been identified as children, pregnant women (approximately 20 times more like to be infected), the elderly, persons with weakened immune systems, persons with cancer, diabetes, kidney disease, persons with AIDS (almost 300 times more likely to be infected), persons who take glucocorticosteroid medications, and the elderly (www.cdc.gov, 2005). In many cases the susceptible population is the group that will be more likely to be consuming high risk foods such as frankfurters or soft unripened cheeses.

The current level of listeriosis in the United States is estimated to be 0.27 cases per 100,000 individuals per year, based on the 2004 U.S. FoodNet surveillance data (Morbidity Mortality Weekly Report, 2004).

Most cases of listeriosis during pregnancy occur in otherwise healthy women. Symptoms that are presented in pregnant women may be non-specific and are often mild. Infection of the fetus is thought to be mainly through the placenta, which can lead to premature delivery, spontaneous miscarriage, stillbirth, or serious illness in the infant. Although listeriosis may occur at any time during pregnancy, it is most frequently documented during the third trimester (Mylonakis et al., 2002). Transplacental infection of the unborn fetus likely causes bacteremia, which leads to the presence of *L. monocytogenes* in the fetus' urine. This urine is then discharged into the amniotic fluid, which in turn is aspirated by the fetus. This then leads to involvement of both the respiratory and gastrointestinal tracts. Newborns (early onset) infected with *L. monocytogenes* present symptoms of respiratory distress, heart failure, difficult and forced respiration, cyanosis, refusal to nurse, vomiting, convulsions, soft whimpering, early discharge of meconium, and mucus in stools. In early onset infections with *L. monocytogenes* involves numerous organs (spleen, adrenal gland, esophagus, posterior pharyngeal wall, and tonsils). Subepithelially and in the lymph nodes, thymus, myocardium, testes, bone marrow and skeletal muscles granulomas may be present. Late-onset neonatal listeriosis disease presents itself as meningitis more frequently than in early-onset disease. The route of infection in late-onset listeriosis is not well understood. It is thought that the infection is acquired during passage through the birth

canal; however, there have been some reports of late-onset disease following cesarean delivery.

The infective dose depends on three factors, the environment (or the food matrix), the pathogen (virulence), and especially the immunological status of the host. The dose-response relationship for this pathogen is unknown. This is due to the high case fatality rate (20%), which makes human feeding studies unacceptable (Gombas et al., 2003). Hitchins in 1996 estimated that the infectious dose was greater than 10^3 CFU and that low levels of *L. monocytogenes* (levels that did not induce listeriosis) were being consumed almost 100 times per year. This claim is further substantiated by the fact that more than 90 percent of adults possess immune lymphocytes (Hof, H., 2003). It is also estimated that around five percent of the healthy population harbors the bacterium in their gastrointestinal tract (Donnelly, 2006). There are three serotypes (4b, 1/2a, and 1/2b) that account for 96 percent of the human *L. monocytogenes* infections in the United States (ILSI, 2005). *L. monocytogenes* accounts for four percent of all hospitalizations and 28 percent of all deaths from foodborne disease in the United States (Mead et al., 1999). In foods the most commonly occurring species are *L. monocytogenes and L. innocua.* The serotype of *L. monocytogenes* most often associated with foodborne outbreaks is serotype 4b (Ryser and Marth, 2007).

Foodborne Sources of *Listeria monocytogenes*

A common feature of many foods implicated in foodborne listeriosis is that they are ready-to-eat. Most of these ready-to-eat foods were cured, pasteurized or cooked

during processing, indicating post-processing contamination. While evidence suggested that *L. monocytogenes* could be transmitted via food, the first documented foodborne listeriosis in the United States did not occur until 1979 when vegetables prepared in a Boston hospital were implicated (Ramaswamy, et al., 2007). However, Cliver (1990) suggests that the first recorded outbreak of listeriosis actually occurred in post World War II Europe with milk being the food vehicle implicated. The first major outbreak of listeriosis occurred in 1981, and the probable food vehicle was determined to be coleslaw which had been made using cabbage from a field fertilized using manure of *Listeria* positive sheep. Prior to this, *Listeria* was considered primarily an animal pathogen, and that it was transmitted via direct contact with infected animals (Donnelly, 2006). The first laboratory-confirmed association of a meat/poultry product with invasive listeriosis occurred in 1988, when a case was linked to the consumption of contaminated turkey franks (Morbidity Mortality Weekly Report, 1989). The patient was hospitalized with sepsis caused by *L. monocytogenes* serotype 1/2a, and reported daily consumption of turkey frankfurters. *L. monocytogenes* serotype 1/2a was isolated from frankfurters obtained from the patient's refrigerator as well as from unopened packages of the same brand of frankfurters from obtained from the local store. The isolates from the food and patient were of the same electrophoretic type (Wenger et al., 1990). Based on these data USDA-FSIS and the CDC began an environmental investigation at the processing facility to identify both the potential routes of contamination and strategies to prevent product contamination. *L. monocytogenes* was isolated from six of the seven finished product samples tested from five lots which matched the case isolate. Sampling of the production line revealed an increased contamination frequency of samples following the mechanical

removal (peeling) of the cellulose casing, subsequent swab samples from the conveyer belt onto which the peeled frankfurters dropped yielded the same subtype (Wenger et al., 1990). *L. monocytogenes* serotype 1/2b, was isolated from environmental and product samples in the room where the cooked frankfurters were cooled prior to peeling. This environmental investigation provided strong evidence that the source of contamination was the processing environment itself, and that the contamination occurred at the peeling step after the heat treatment, which would have killed the pathogen. Further, this study revealed that over a four month period this subtype was repeatedly isolated from the plant suggesting its persistence in the processing environment. The presence and persistence of *L. monocytogenes* in processing environments has proven to be a significant challenge to regulators and the food industry alike. The contamination of ready-to-eat meat and poultry products after preparation of the finished product, but before packaging, led to three multistate listeriosis outbreaks over the following 12 years (Ryser and Marth, 2007). This investigation provided evidence that even low level finished product contamination needs to be prevented, since *L. monocytogenes* proliferates at storage conditions and in this case appeared to have increased several logs during home refrigeration of the implicated frankfurters.

Over the years many types of foods have been associated with *L. monocytogenes* outbreaks. From 1979 - 2002 there have been 466 documented cases of listeriosis from food products (Food and Drug Administration, 2003). The food vehicles that were identified for these outbreaks were raw vegetables, cheese, pasteurized fluid milk, raw cheese, ice cream, salami, raw eggs, butter, frozen vegetables, frankfurters, deli meats, and pate. Varma et al. (2007) found 11 exposures to be associated with an increased

likelihood of listeriosis, including; eating hummus, eating Mexican-style cheese, having a soft cheese (brie, camembert, etc.) in the refrigerator, eating melons, eating "ice milk", and buying any soft cheese. Vit et al. (2007) linked a 2006 listeriosis outbreak in the Czech Republic to mature cheese sold in two different food store chains, and to a mixed salad containing salami, cheese, vegetables, and dressing. Jorgensen and Huss reported in 1998 a prevalence rate of *L. monocytogenes* in cold-smoked fish to be between 34 percent and 60 percent. These researchers also found in heat-treated and cured seafood the *L. monocytogenes* contamination level to be between four percent and 12 percent, indicating that these products could pose a significant health risk if consumed by someone in the at risk population. Wang and Muriana (1994) found that out of 20 brand of retail frankfurters, 19 (93 samples) had a 10 percent incidence of listeriae, and an eight percent incidence of *L. monocytogenes.* With one of the brands tested exhibiting a 71 percent incidence of *L. monocytogenes* and an overall *Listeria* spp. of 83 percent. Further, these researches found *Listeria* in the purge at a level of 1-3 CFU/ml, but not in the frankfurters themselves, indicating post-processing contamination.

From January 1994-October 2006, approximately 175 Class I voluntary recalls were issued for RTE and cooked meat contaminated with *L. monocytogenes*; 74 for deli meats, 42 for sausages, 37 for frankfurters and 22 for other products (ham salad, sandwiches etc.) (Ryser and Marth, 2007). Table 1. Shows a complete list of Class I recall issued in the United States from 1991-January 2009 for cooked and RTE meat products found to be contaminated with *L. monocytogenes* (Food Safety Inspection Service, 2009).

Table 1. Chronological List of Recalls (Class I or Voluntary) Issued for Various Food Products Contaminated with *Listeria monocytogenes* 1991-January 2009.

Product	**Date Recall Initiated**	**Origin**	**Quantity in lbs**
Sandwiches	1991	LA	NA
Ham Salad	1991	MN	460
Sandwiches	07/03/91	LA	NA
Skinless Frankfurters	1991	MI	3,700
Ham Salad	1991	WV	600
Frankfurters	03/20/92	CT	3,578
Sandwiches	06/18/92	LA	NA
Sandwiches	09/25/92	TN	NA
Frankfurters	01/25/94	CA	1,220
Frankfurters	02/09/94	MI	1,600
Beef Cooked Salami	02/23/94	NY	1,268
Linguica Sausage	03/21/94	RI	100
Ham, Sliced	03/28/94	NE	3,950
Ham Salad	04/12/94	IN	1,105
Ham, Cooked, Sliced	05/05/94	MI	844
Smoked Sausage	05/15/94	MS	250
Frankfurters	10/03/94	NE	5,500
Frankfurters	10/14/94	NY	432
Ham, Sliced	10/20/94	NM	3,920
Smoked Polish Sausage	01/10/95	MN	36
Beef Franks	01/18/95	PA	1,000
Buffalo Franks	03/03/95	CO	260
Frankfurters	05/30/95	NY	3,780
Hot Dogs (Franks)	07/06/95	BC, Canada	11,420
Bologna Ring	07/24/95	PA	200
Sandwiches	09/08/95	MI	NA
Beef Wieners	10/27/95	AB, Canada	3,510
Hot Dogs, Pork	11/06/95	AB, Canada	4,320
Sandwiches	02/16/96	LA	NA
Cooked Beef Roast	03/13/96	NE	487
Roast Beef, Sliced	04/03/96	IL	720
Cooked Beef	06/14/96	TX	2,608
Extra Hot Beef Jerky	06/20/96	BC, Canada	360
Ham, Chunked and Formed	10/24/96	OH	400
Sliced Ham	12/09/96	NH	52
Sandwiches	01/22/97	MS	NA
Dry Sausage	10/03/97	NY	347

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
Filzette Salami	12/18/97	MO	507
Frankfurters	03/19/98	NY	1,440
Ham Steak	04/17/98	MO	635
Dry Sausage	06/04/98	CA	272
Frankfurters	10/22/98	FL	1,734,002
Smoked Beef Strips	10/29/98	AR	3,600
Hot Dogs/Packaged Meats	12/22/98	MI	35,000,000
Luncheon Meat	01/15/99	WI	38,312
Sliced Ham	01/22/99	OH	348
Various	01/22/99	AR	35,000,000
Mortedella	02/05/99	ON, Canada	456
Hot Dog, Bockwurst	02/05/99	WA	1,545
Head Cheese	02/17/99	IL	2,586
Frankfurters	02/18/99	GA	4,460
Pasta with Sausage	03/02/99	IL	1,923
Frankfurters	03/18/99	NJ	18
Luncheon Meats	05/14/99	NC	16,392
Weisswurst	05/28/99	PA	60
Wieners, Frankfurters	06/01/99	ND	150
Franks, skinless	06/04/99	IL	1,285
Frankfurters	06/18/99	HI	9,620
Bacon Chips	07/27/99	IN	126,739
Ham, Sliced/Whole	07/29/99	MI	200
Roasting Sausage	07/30/99	NJ	200
Hot Dogs	07/30/99	PA	200
Chorizos	08/26/99	PR	1,640
Sausage	08/27/99	MA	3,720
Frankfurters, Beef	10/13/99	NY	2,100,000
Smoked Sausage	11/12/99	LA	1,270
Hot Dogs	11/12/99	HI	312
Hot Dogs	11/18/99	PA	1,020
Polish Sausage	11/19/99	MD	800
Beef Franks	12/09/99	PA	4
Luncheon Meat	12/14/99	NJ	900
Pâtés and Mousses	12/17/99	NY	10,064
Roast Beef	01/14/00	AZ	600
Dry Sausage	01/28/00	IL	200
Cooked Corned Beef and Ham	03/01/00	MI	80
Hot Dogs	03/15/00	NY	400
Franks	03/24/00	PA	34,500

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
Roast Beef	04/06/00	UT	13,351
Sausage, Various	04/13/00	OH	850
Sliced Cooked Meats	04/26/00	MI	180
Sliced Cooked Meats	05/03/00	MI	215
Sliced Cooked Meats	05/05/00	MD	450
Pork Cretons Spread	05/10/00	ME	210
Frankfurters	05/11/00	HI	1,125
Sausage, Various	05/12/00	MS	5,900
Salami, Westphalian Ham	05/19/00	BC, Canada	400
Franks	05/24/00	MI	2,870
Sliced Cooked Meats	05/24/00	AL	45
Franks	05/29/00	NC	15,000
Sliced Ham	06/07/00	MO	60
Beef Bologna	06/07/00	NY	2,200
Ham	06/14/00	CA	1,800
Sliced Luncheon Meat	06/11/00	HI	270
Beef Jerky	07/06/00	CT	125
Hot Dogs, RTE Deli Meats	08/06/00	NY	19,000
Sliced Luncheon Meat	08/08/00	ID	380
Wieners	10/03/00	TN	900,000
Polish Sausage	10/04/00	MN	240
Country Ham	12/20/00	KY	10,400
Mexican-style Meat Products	01/30/01	IL	NA
Hungarian Salami	02/28/01	NJ	3,700
Sliced, Cooked Beef	03/22/01	IL	1,570
RTE Beef Sausage	03/28/01	NY	7,800
RTE Meat and Poultry Products	04/12/01	OK	14,500,000
RTE Bratwurst	07/01/01	WA	100
Cooked Smoked Ham	07/05/01	IA	70
RTE Pork Andouille Sausage	09/06/01	WA	20
Cooked Pork and Corned Beef	09/26/01	IN	NA
Cooked Beef Products	10/05/01	IL	5,600
Cooked Beef Products	10/10/01	IL	5,000
Luncheon Meat	10/31/01	IA	189,000
Cooked Roast Beef Products	12/12/01	IL	700
Bratwurst	12/19/01	MO	115
Sausage Patties	12/20/01	NC	3,300
Beef and Pork Sausage Products	01/15/02	TX	2,500
Pork Sausage Products	01/18/02	FL	150
RTE Semi-Dry Sausage	02/06/02	NY	1,800

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
Dried, Seasons Beef	02/12/02	CA	22
Chopped, Cooked Ham	02/18/02	CA	200
Beef and Pork Sausage	03/05/02	TX	360
RTE Souses Loaf	04/17/02	VA	190
Frankfurters and Hot Dogs	04/25/02	OH	140,000
RTE Ham	05/07/02	NV	300
Frankfurters and Bologna	05/23/02	NY	77,000
Sliced Ham	06/17/02	MO	10
Cured Ham	06/21/02	NY	2,300
Sausage Products	06/25/02	MN	250
Sliced Pork Hocks	07/03/02	NY	100
RTE Braunschweiger	07/19/02	NY	65
Sausage	07/27/02	PA	22
RTE Italian Loaf	08/10/02	NY	1,300
Sliced Corned Beef	08/14/02	FL	19
Sausage Product	08/16/02	Fl	500
Cooked, Boneless Ham	08/28/02	AR	2,200
Cooked, Boneless Ham	08/30/02	AR	6,525
Imported RTE Sausage	09/04/02	PA	1,035
RTE Prosciutto Ham	09/04/02	CA	510
Cooked and Cured Beef Loaf	09/17/02	PA	185
RTE Ham	10/08/02	IN	95
Pork Dumplings	10/11/02	HI	150
RTE Pork Shoulder	11/20/02	PR	6,800
RTE Pork Luncheon Meat	11/26/02	IN	210
Cooked Pork Products	11/27/02	VA	540
RTE Souse Products	12/03/02	MS	500
Pork Sausage	12/04/02	FL	8,600
Pork Sausage	12/12/02	FL	200
Chicken Frankfurters	01/03/03	NY	26,400
Cooked Beef Products	01/22/03	SD	2,100
Cooked Pork Shoulder	01/28/03	PR	490
Cooked Pork Shoulder	01/30/03	PR	500
Smoked Pork Chops	03/06/03	PA	11
RTE Fully Cooked Pork and Veal Bologna	03/22/03	NY	330
Cooked Beef Sausage	05/05/03	NC	180
RTE Chicken Salad	05/05/03	IL	400
RTE Thai-Style Noodle Salad Containing Chicken	10/04/03	NJ	270

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
RTE Luncheon Meat	10/10/03	CA	550
RTE Meat and Poultry	10/12/03	MA	9,230
Beef Products	10/24/03	WA	200
Cooked Roast Beef	11/18/03	NE	110
Cooked Diced Chicken Breast	11/25/03	GA	7,500
Chicken Salad	12/11/03	FL	2,700
Frozen Beef	01/11/04	IL	5,190
RTE Meat Products	01/28/04	PA	52,000
Beef and Pork Frankfurters	02/17/04	NY	540
Fully Cooked Boneless Ham	03/16/04	GA	713
RTE Fully Cooked Bologna	04/05/04	PA	100
Fresh Deli Meat and Cheese Trays	04/12/04	IL	135
Scrapple	06/03/04	DE	350
Beef Jerky	06/29/04	PA	130
Frozen, Fully Cooked Chicken Products	07/01/04	NC	404,730
Frozen, Fully Cooked Chicken Products	07/21/04	GA	36,980
Beef and Pork Products	07/27/04	NJ	500
Wieners	08/04/04	UT	5,360
Fully Cooked Ham	08/10/04	NY	422
RTE Chicken Products	11/03/04	MD	1,275
Chicken Products	01/31/05	NY	5,760
RTE Ham	02/09/05	TN	47
Cooked Pork Products	02/28/05	LA	1,120
Chicken Salad	03/15/05	NY	250
Chicken Products	03/25/05	CA	12,500
Chicken Wrap Sandwiches	04/05/05	FL	3,316
Various Sausage Products	04/05/05	MI	5,117
Smoked Turkey and Pork Products	04/11/05	NY	39,000
Various Sausage Products	04/11/05	GA	10,700
Pork Blood Sausage	04/19/05	CA	40
Various RTE Meat Products	04/21/05	MO	1,077
Deli Meat Wraps	04/26/05	TX	191
Chicken Breast Wraps	04/28/05	NY	385
RTE Ham	04/29/05	KY	29,000
Various RTE Meat Products	04/30/05	Nationwide	363,332
RTE Chicken Salad	06/15/05	NY	5,065
Spanish Brand Sausage (Primera Chorizo)	06/26/05	NJ	720
Natural Proportion Cooked Chicken Meat	07/21/05	GA	170

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
Meat and Poultry Products	07/29/05	Nationwide	93,200
Barbeque Beans with Beef and Chicken Salad	08/31/05	OK	23,435
Chorizo, Blood Sausage and Blood Pudding	09/15/05	NY	890
Chicken Frankfurters	09/20/05	NY	23,040
Cooked Country Hams	09/28/05	VA	165
Cooked Chicken Sausage Products and Beef Wieners	10/02/05	IL	1,000
RTE Meat and Poultry Products	10/22/05	MA	11,200
RTE Beef Products	10/31/05	NY	2,263
RTE Chicken Products	11/08/05	CA	275
Chicken Salad Products	11/10/05	PA	5,523
Turkey, Ham, Bologna and Chicken Lunch Makers Meals	12/01/05	MO	2,800,000
Pork Barbeque	02/18/06	NC	30
Dried Beef Products	03/23/06	TX	100
Ham Salad	04/05/06	ME	92
Hot Dogs	07/22/06	CA	525
Pork Products	10/12/06	OH	1,178
Ham and Turkey Products	11/24/06	OH	46,941
Hog Head Cheese	01/03/07	LA	290
Sausage Products, Franks	01/05/07	CO	15,514
Semi-Boneless Ham Steaks	02/27/07	NV	930
Chicken Breast Strips	02/28/07	SC	2,800,000
Grilled Balsamic Flavored Chicken Breast	06/05/07	NY	140
RTE Chicken Products	06/29/07	TN	2,768
Chicken and Pasta Product	10/09/07	CT	70,400
Beef Patty Products	12/25/08	NJ	88
Frozen Chicken Entrée	03/02/08	IL, IN, MI, OH	2,184
Frozen Chicken Entrée	03/03/08	AK, ID, MT, OR, UT, WA	10,1368
Frozen Chicken Entrée	03/04/08	Nationwide	3,780
Various Fresh and Frozen Meat and Poultry Products	03/04/08	CT, FL, GA, MA, MN, NJ, NY, PA, SC, WI	6,970
Various Fresh and Frozen Meat and Poultry Products	05/03/08	Nationwide	286,320

Table 1. Continued.

Product	Date Recall Initiated	Origin	Quantity in lbs
Pork Blood Sausage	05/12/08	CA	290
Various RTE Chicken Products	06/09/08	CA	130
Various RTE Chicken Products	08/05/08	MA	285
Various Fully Cooked Pork Products	08/10/08	HI	4,535
RTE Turkey Burrito Wrap	10/30/08	IL	16
Hot Dog Products	11/08/08	AL, FL, GA, MS, TN	28,610
RTE Frozen Beef Sandwich	11/28/08	GA, CA, CL, IL, NY	5,250
Sporessata Sausage	12/19/08	OH	1
Karkow Sausage	12/25/08	MO	750
Bacon Bit Products	01/03/09	CA, CO, FL, SD, TX, WI	3,590
RTE Burritos	01/06/09	CO	172

Regulatory Control/Action

In 1987 the USDA/FSIS began to monitor *Listeria monocytogenes* in meat products (domestic corned beef, cooked corned beef, massaged corned beef and imported cooked meats). Then in 1988 this program was expanded to include a greater range of products including meat and poultry salads and spreads (Crawford, 1989).

In the 1990s, the CDC and FSIS began to investigate an outbreak of foodborne illness in which both frankfurters and deli meats were indicated as the possible vehicle of infection. These investigators were eventually able to isolate the outbreak strain of *L. monocytogenes* from a package of unopened frankfurters. In all 101 illnesses were reported, along with six stillbirths or miscarriages and 15 adult deaths.

This outbreak resulted in the FSIS notifying manufactures that they need to re-evaluate their HACCP plans to assure that risk of contamination with *L. monocytogenes* was adequately addressed.

It was recognized that *L. monocytogenes* has caused foodborne disease from food products regulated by both the FDA and USDA; therefore, a zero tolerance policy was put into place for this bacteria. The USDA-FSIS has required that federally inspected establishments that produce ready-to-eat (RTE) meat and poultry products implement one of the following for the control of *Listeria monocytogenes* (FSIS Directive 10,240.4, 2009).

I. Use of a post-lethality treatment (which may also be the antimicrobial agent or process) that reduces or eliminates microorganisms on the product AND an antimicrobial agent or process that suppress or limits the growth of *L. monocytogenes.*

II. Use of either post-lethality treatment (which may be an antimicrobial agent or process) that reduces or eliminates microorganisms on the product OR an antimicrobial agent or process that suppress or limits the growth of *L. monocytogenes.*

III. Use of sanitation measures only.

In 2003 fifteen trade organizations submitted a petition requesting that the FDA amend its zero tolerance regulation and to establish a regulatory limit of 100 CFU/g for foods that do not support growth of *L. monocytogenes.* The FDA then announced that is pathogen-monitoring program would concentrate on high-risk foods, including frankfurters (Anonymous, 1996).

Biofilm Formation

Many factors influence the growth of microorganisms in food processing environments, including oxidation-reduction potential, pH, temperature, moisture,

nutrients, presence or absence of inhibitors, interactions between microorganisms in a population and time. Transfer of microbes present in a food processing factory into niches that may be inaccessible to cleaning and sanitation can occur via air, water, tools, workers, traffic and other means (Ryser and Marth, 2007). *Listeria* cells may attach to environmental surfaces with membrane bound structures (flagella, proteins etc.) given enough contact time between the cells and the surfaces. Further, if sufficient nutrients, time, and moisture are available to the microbe biofilms may develop.

Todhanakasem and Young (2008) found that colonization of surfaces with *L. monocytogenes* occurs in 4 stages; Stage 1is initial attachment of the bacteria, Stage 2 involves microcolony formation within 24 hours, Stage 3 has complete tertiary structure maturations occurring in 48 hours, Stage 4 shows approximately 12 hour cycles of dissociation and biofilms regrowth. Attachment of *L. monocytogenes* has been attributed to fibrils, the presence of flagella, synthesis of exopolysaccharides and hydrophilic interactions (Ryser and Marth, 2007). The attachment process is significantly influenced by temperature, which likely leads to the modification of the cells surface properties such as hydrophobicity and attachment factors available to the bacterium (Di Bonaventura et al., 2008). Wong (1998) found that of the conditions tested 6°C and RH of 75.5% to be the most favorable for the survival of *L. monocytogenes* biofilms. However, the pathogen survived at all tested combinations, 25°C and 32.5% RH, 25°C and 75.5% RH, 6°C and 32.5% RH. These conditions are commonly found in food processing environments.

The survival of *L. monocytogenes* in the food processing environment is prolonged because if its ability to establish biofilms on the surfaces of equipment.

Biofilm survival is affected by temperature, relative humidity (RH), surface on which it is attached and soil (Wong, 1998). The highest associated incidences of *L. monocytogenes* in the food processing environment are in wet locations, floor drains and conveyor belts being prime examples. Levels of *L. monocytogenes* in floor drains is reported to range from 3.6 log CFU/cm^2 to 7.5 log CFU/cm^2 (Zhao et al., 2006). The establishment of biofilms by pathogenic bacteria is floor drains in food processing plants is believed to protect against effective cleaning regimens and to reduce the efficacy of bactericidal treatments. Zhao et al. (2006) found that *Lactococcus lactis* subspecies *lactis* and *Enterococcus durans* to inhibit the growth of *L. monocytogenes* and to reduce (up to 4.1 log CFU/cm^2) biofilms in floor drains. These findings show that the application of competitive microflora can greatly reduce the levels of *L. monocytogenes* and may be an important strategy in the control of biofilms in the food processing environment.

L. monocytogenes can attach to many surfaces commonly found in food processing environments including stainless steel, glass, wood, Buna-N rubber, porcelain, iron, plastics, polypropylene, waxed cardboard, and paper (Krysinski et al., 1992; Mafu et al., 1990; Mosteller and Bishop, 1993; Stanfield et al., 1987). As a result of this ability equipment surfaces, conveyor belts, floor sealant, and drains may all be potential reservoirs for *Listeria* spp. in a food processing plant and may consequently lead to secondary food contamination. Lunden et al. (200) found that strains of *L. monocytogenes* that are persistent in food processing environments form thicker biofilms than isolates found only sporadically. This gives support to the idea that biofilm formation is important for the survival of *L. monocytogenes* in the food industry. Interestingly, Di Bonaventura et al. (2008) found no difference in biofilm formation with

regard to linage, source (food or environment), and origin (fish versus meat). These researchers also found that the ability of *L. monocytogenes* to attach to surfaces is also not dependent on serotypes. This is in contrast to Romanova et al. (2007) who found that *L.* monocytogenes strains belonging to serotype 1 were significantly better biofilm formers than strains belonging to those of serotype 4 (P=0.0003). These data suggest that the ability to *L. monocytogenes* to form adherent colonies is a complex process, and may dependent on a not yet defined characteristic of this pathogen.

The physiology and structure of planktonic cells differs from those of adherent cells. *L. monocytogenes* in the stationary phase in biofilms show changes in morphology, changing from rod to cocci as the population ages (Tremoulet et al., 2002). Interestingly this pathogen grows more slowly when in a biofilm than when grown as planktonic cells. Not surprisingly proteins associated with stress response, quorum sensing regulation, DNA repair, cell multiplication and carbohydrate metabolism are up-regulated during biofilm development (Tremoulet et al., 2002). Flagellin synthesis, a flagellar protein, is decreased during biofilm growth (Tremoulet et al., 2002). This suggests that while flagella appear to be important for initial attachment and colonization of surfaces, they may not be useful for the formation of the biofilms structure.

Analysis of *L. monocytogenes* microscopically shows an increase in cell density over time while enumeration on agar indicates a constant colony count (Tremoulet et al., 2002). These researches hypothesized that this difference in count by the two enumeration methods may be due to an increase in noncultivable or dead *L. monocytogenes* as the biofilms ages. These dead cells may contribute to the increased

resistance of biofilms to cleaning, disinfecting and sanitizing, by providing a layer of protection for the living cells.

It is widely accepted that biofilms are more resistant to cleaning procedures and sanitizers when compared to planktonic cells. Susceptibility to disinfection appears to be dependent of the surface to which *Listeria* are attached (Adriao et al., 2008). Romanova et al. (2007) fond that the minimum inhibitory concentration (MIC) of benzalkonium chloride, a commonly used industrial sanitizer (quaternary ammonia) increased approximately 1000-fold in comparison to the MIC of planktonic cells. These researchers found that *L. monocytogenes* biofilms were eliminated from stainless steel surfaces using 0.25% (w/v) of hypochlorite within 15 minutes, while a 1% (w/v) solution was necessary for the removal of adherent *L. monocytogenes* from polystyrene. Adrido et al. (2008) also found that attached *L. monocytogenes* were able to survive at low pH (pH 3.5) more readily than planktonic *L. monocytogenes* strains. These findings highlight the difficulty in removing adherent cells as well as the increased survivability of *Listeria* in biofilms. Suihko et al. (2002) found the prevalence of *L. monocytogenes* in the meat, poultry, and seafood industry to be approximately 9%). Their research also showed that after normal cleaning procedures and during processing, this pathogen could be recovered from a variety of sources, with conveyor belts and floor drains being the highest levels of contamination. Pan et al. (2006) found that *L. monocytogenes* biofilms on stainless steel and Teflon coupons in a simulated food processing environment initially decreased in numbers for one week then adapted to the environment and increased in number and had an increase in resistance to sanitizers. These researchers also found that when these cells were detached from the biofilms, they did not show

increased resistance to sanitizers. This may be due to the protection of the exopolysaccharide produced by the biofilms.

Incidence and Control of *Listeria* in the Food Processing Environment

In 1995 the World Health Organization (WHO) performed a survey in Europe which indicated that almost 25% of the foodborne outbreaks were due to recontamination of food products. The presence of pathogens in prepared foods was significantly linked to insufficient hygiene (1.6%), cross-contamination (3.6%), processing or storage in inadequate rooms (4.2%), contaminated equipment (5.7%) and contamination by personnel (9.2%). Raw materials or ingredients added to processed products have been identified as sources of contamination. Contamination of ready-to-eat (RTE) products by pathogens in raw products, as a result of inadequate hygiene is a well recognized issue. Because *L. monocytogenes* is ubiquitous in nature it is possible that raw material coming into the production plant may be contaminated. *L. monocytogenes* has been reported at levels from 4-2.1 x 10^4 CFU/g in raw meat (Ryser et al.). FSIS in 1995 (Anonymous, 1995) conducted a survey that found that, based on 600 1-lb samples of ground beef, from 661 plants, there was an 18% incidence rate.

Recontamination of food products due to unclean, inadequately, or insufficiently cleaned pieces of equipment has been identified as the source of pathogens are numerous. Rorvik et al. (1995) demonstrated that food contact surfaces of processing equipment allowed for the transfer of *L. monocytogenes* during processing. The strongest evidence that *L. monocytogenes* and other *Listeria* spp. enter commercially processed foods as post

processing contaminants arises from the fact that apparently healthy, non-thermally injured cells are recovered from many thermally processed meat, poultry, seafood, and dairy products. As well as the fact that these organisms have been found in virtually all processing plants that have had a *Listeria*-related recall (Ryser and Marth, 2007). To date, no *Listeria*-related commercially prepared food products have been unequivocally linked to the inadequacy of minimum required heat treatments, despite this pathogen's greater heat resistance when compare to other vegetative microbes (Ryser and Marth, 2007). Further, Zaika and co-workers (1990) found that by using a commercial, standard step-heating schedule (70 minutes for frankfurters to reach an internal temperature of 160°F) kills the *L. monocytogenes* at the levels encountered in raw meats ($\leq 10^3$ CFU/g).

Defective or soiled packaging may also be a source of recontamination. Defective seams and seals for instance can cause micro-leaks that may allow access of a variety of pathogens (Reij and Den Aatrekker, 2004). Ryser and Marth (2007) suggest that the ability of *Listeria* to attach to objects with different surface properties may mean that packaging materials need to be considered as potential contamination sources.

Airborne microorganisms are usually associated with dust particles or water droplets (aerosols) and transmission due to airflow. In processing environments distribution of pathogens is probably due more to the creation of aerosols than dust. Rorvik et al. (1997) described the impact of *L. monocytogenes* aerosols from drains and its subsequent dissemination through the processing facility. Floor drains in food processing facility are a particularly important niche for the persistence of listeriae and can be a point of contamination in the processing plant environment and therefore

possibly in food products. Other studies have looked at the creation of aerosols created from drip trays, cooling units (Goff and Slade, 1990) or through high pressure hosing (Anonymous 1999). Research presented by De Roin et al. (2003) suggested that frankfurters exposed to dust contaminated with *L. monocytogenes* could result in measurable recovery of the organism.

Although it may be possible for *Listeria monocytogenes* to enter the food chain at nearly any point, the food processing environment appears to be of particular importance for introduction of this pathogen into the food system (Mathews, 1928). The use of Sanitary Standard Operating Procedures (SSOPs), Standard Operating Procedures (SOPs) and Good Manufacturing (GMPs) practices are critical to the production of a safe food product. Pathogens may access food processing environments through raw materials, personnel or mobile equipment, through leaks and openings in buildings, or through pests. Pathogens such as *L. monocytogenes* may become established in the processing environment and find niches where they can survive for long periods of time (months to years). Crack and crevices in floors and walls, drains, and interfaces between the floor and equipment may form such niches.

Microflora that develops in niches is directly impacted by the water activity of the niches (Faust and Gabis, 1988). Faust and Gabis (1988) showed that disruption of these niches may result in direct or indirect contamination of the food product. These researchers found the probability of product contamination to be affected by several variables (1) Proximity of microbial growth niches to product line(s), (2) number of niches, (3) spatial relationships of niches to the product line(s), (4) microbial populations

within niches, (5) extent of disruption and (6) exposure of the product line(s) to the environment.

Eradication of *L. monocytogenes* from contaminated processing machines and lines has proven to be difficult and requires special measures including targeted cleaning and disinfecting procedures. The cleaning and disinfecting procedures for food processing equipment involves mechanical, chemical and thermal energy. Mechanical cleaning is the most effective way to detach biofilms form food contact surfaces (Gibson et al., 1999). Adherence to food contact surfaces in the processing environment is important for the survival of bacteria, adherence increases the resistance of the pathogen to mechanical and chemical stresses (Eginton et al., 1998; Frank and Koffi, 1990; Ronner and Wong, 1993). Persistent *L. monocytogenes* strains have been shown to be more adherent to stainless steel surfaces after short contact time when compared to non-persistent strains (Lunden et al., 2000).

Control of *L. monocytogenes* in the processing plant is dependent upon a number of elements: control of the traffic flow (raw and processed material do not cross paths), training of employees, sanitary equipment and facility design, cleaning and sanitation procedures, testing of the production facility, a low moisture environment, and implementation and proper use of a Hazard Analysis and Critical Control Point (HACCP). Among these factors moisture is the most critical for the organism (Faust and Gabis, 1988) and one of the easiest to control. Control of *L. monocytogenes* is not limited to just the processing facility. Product formulation (when possible) should include the use of an antimicrobial, in-package controls should also be used where

applicable such as irradiation and steam pasteurization. It is also important to avoid temperature abuse of the product and care should be taken to monitor temperature throughout the processing and distribution chain. Freezing and frozen storage prevent the outgrowth of *L. monocytogenes.* Due to the "zero tolerance" level of acceptance in food products for *L. monocytogenes*, testing has become even more important for control of this pathogen. There are many different methods for testing for *Listeria* in different food products. Tests should be rapid, accurate, precise, and validated as well as internationally acceptable. Testing should not be limited to the food product and raw material alone; environmental testing of the processing plant should also be done, paying close attention to food contact areas, floors, drains, and vents.

Antimicrobials

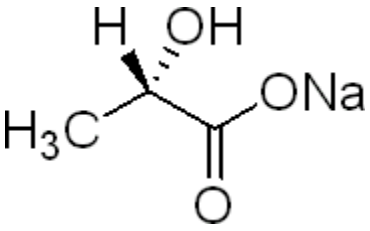
Antimicrobials are defined as any of a large variety of chemical compounds and physical agents that are used to destroy microorganisms or to prevent their development. Antimicrobials include both naturally and synthetically produced chemicals. Organic acids and their salts are frequently incorporated into foods as acidulants or preservatives. Acid sprays and acid dips are used to inactivate *L. monocytogenes* on food surfaces. There are many organic acids used as antimicrobials in foods such as lactic acid, sodium acetate, potassium lactate, sodium lactate, acetic acid, and propionic acid. Bedie et al. (2001) found that inclusion of antimicrobials in frankfurter formulation did not affect cooking yields, moisture content, or fat content. In addition inclusion of antimicrobials was found to not affect over all acceptability and flavor in frankfurters (Barmpalia et al. 2004). Islam et al. (2002) found that in all instances the concentration of antimicrobials

applied directly to the surface of frankfurters, was indirectly proportional to the amount of outgrowth seen.

Sodium Lactate

Sodium lactate (SL) is derived from lactic acid, which is naturally present in animal tissue, and is the sodium salt of lactic acid (21CFR§184.1768). The chemical structure of sodium lactate is shown in Figure 1. Lactates act as a bacteriostat by increasing the lag phase of microorganisms. The mechanism of action of lactate is that it interferes with the metabolism of the bacteria, such as intercellular acidification, interference with the proton transfer across the cell membrane, and feedback inhibition (www.purac.com). Lactate also reduces water activity; this suppresses growth of

Figure 1. Chemical Structure of Sodium Lactate



microorganisms allowing for a longer shelf-life (www.purac.com). Sodium lactate may be applied with no limitation other than current good manufacturing practices (21 CFR§184.1b, 2004) and is a GRAS additive. SL in aqueous form is light yellow in color, has little to no odor, has a pH of 6.5-8.5 (10-60% aqueous solution) at 25˚C, and is stable at normal conditions. Research has shown that the inclusion of SL in the frankfurter formulation at 3% has a bacteriostatic effect, and when applied at 6% had a bactericidal effect even under extended storage conditions (Bedie et al. 2001). In addition to

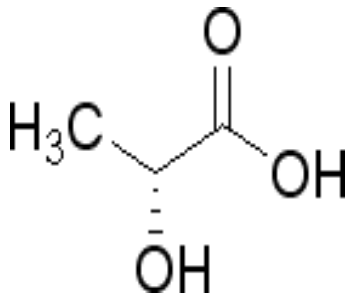
antilisterial effects, Bacus and Bontenbal (1991) found that inclusion of both 3.3 and 6.7 percent liquid sodium lactate in frankfurter formulation also inhibited the natural microflora of the product, thus increasing shelf-life. Their research also showed that addition of two and four percent sodium lactate retarded the outgrowth of *L. monocytogenes* in temperature abused frankfurters up to six days. Application of sodium chloride (2-3%), nitrate (125 ppm), or low temperature enhanced the listeriostatic effect of lactate against *L. monocytogenes* in meat and smoked salmon (Pelroy, 1994; Weaver and Shelef, 1993).

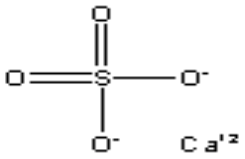
Sodium lactate was also shown to increase palatability when applied at one percent, deceased L* and b* values and increased a* values (Papadopoulos et al. 1991). In contrast to this Brewer et al. (1991) found additions of SL at both two and three percent had no effect on L*, b*, and a* values. Papadopoulos et al. (1991) performed research which showed that addition of sodium lactate did not adversely affect sensory characteristics of beef rounds, however, sodium lactate was found to cause slight throat irritation when applied at six percent. Brewer et al. (1991) also found that SL enhanced pork and salty favors.

Acidified Calcium Sulfate

Acidified calcium sulfate (ACS) is a GRAS additive. ACS is comprised of lactic acid, calcium sulfate, sodium, potassium, ethanol, chloride and water. The chemical structure of some of the components of ACS are shown in Figure 2.

Figure 2. Chemical Structures of the Components of Acidified Calcium Sulfate





Lactic Acid Calcium Sulfate

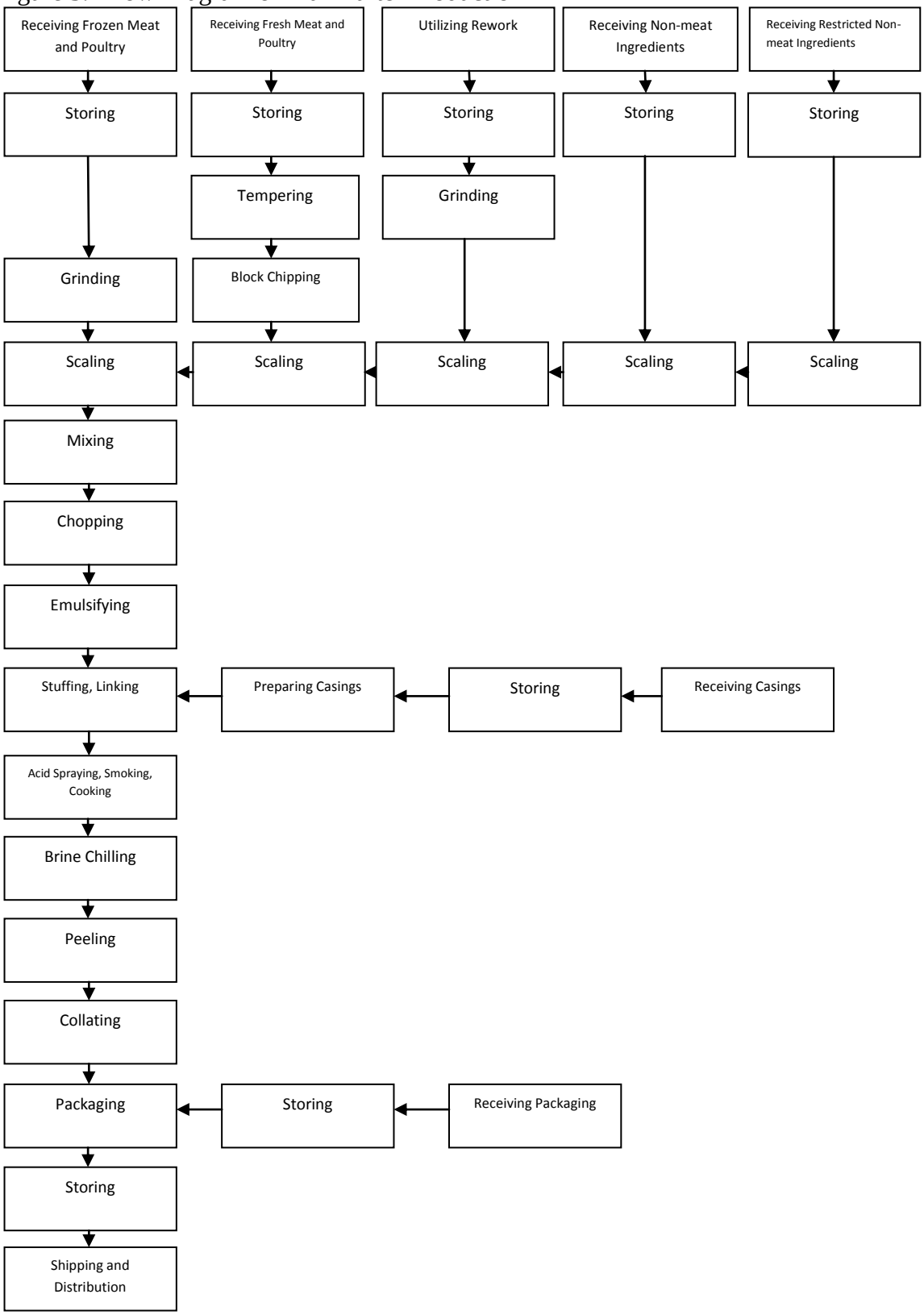
ACS prevents foodborne pathogens from attaching to food and is also an acidulent (www.mionix.com). ACS has a pH range of 1.0-7.0, can be in either a liquid or solid state (powder) for application purposes, is clear to slightly cloudy in solution and is stable at normal conditions. Nunez de Gonzalez et al. (2004) found that application of a ratio of 1:2 ACS to water reduced *L. monocytogenes* levels in frankfurters over a 12 week period, thus showing the bactericidal effect of ACS. These researchers also found that the pH of ACS dipped frankfurters was significantly lower than that of potassium lactate and lactic acid dipped frankfurters. Keeton et al. (2003) found increased levels of astringency, sourness, sweetness, bitterness, saltiness and hardness when using ACS as an antimicrobial dip but only at minutely detectable levels. Keeton et al. (2003) also found that the application of ACS slightly decreased the pH of frankfurters when applied as an antimicrobial dip.

Frankfurters

9 CFR § 319.180 (2004) defines frankfurters as cooked sausages that are comminuted, semi-solid sausages prepared from one or more kinds of raw skeletal muscle meat or raw skeletal muscle meat and raw or cooked poultry meat, and being seasoned and cured, using one or more curing agents. They may or may not be smoked. In accordance with the Federal Standard of Identification (United States Department of Agriculture, 2002), the finished frankfurter shall not contain more than 30 percent fat. Frankfurters may not contain more than 10 percent water. Water or ice, or both, may be used to facilitate chopping or mixing or to dissolve the curing ingredients but the sausage shall contain no more than 40 percent of a combination of fat and added water. These sausage products may contain phosphates. Such products may contain raw or cooked poultry meat and/or Mechanically Separated (Kind of Poultry) without skin and without kidneys and sex glands used in accordance with 9 CFR § 381.174 (2006), not in excess of 15 percent of the total ingredients, excluding water, in the sausage. The finished product may not contain more than 30 percent fat. Non-meat binder and extenders may be used up to 3.5 percent or isolated soy protein may be used up to two percent in frankfurters as per 9 CFR § 412.21(c) (1989).

Frankfurters are an example of small diameter emulsion-type sausages. This type of sausage is considered ready-to-eat. The term meat emulsion is a general term to describe the mixture of protein, fat, and water. Because of the fine texture of the meat the sausage mix is a viscous mass and it is not considered a true emulsion. Therefore, these types of sausages, including frankfurters, are considered to be batter-type sausages. Emulsion-type sausages are basically made from a mixture of finely chopped meat, fatty

Figure 3. Flow Diagram of Frankfurter Production



tissue, and water or ice. Figure 3. shows a schematic drawing of frankfurter production. The formulation of emulsion-type sausages not only contains meat with high water holding capacity, but also contains meat with intermediate water holding capacity, filler meats, extenders, binders, fillers, spices, and nitrites. Filler meats may be added but should not exceed 15-20 percent (Food and Agriculture Organization, 1985). Frankfurters may also contain extenders, fillers, or binders. These are included in product formulation for one or more of the following: improve meat batter stability, improve water binding capacity, enhance texture or flavor, reduce shrinkage, improve slicing characteristics, or reduce costs.

Comminution, the process in which particle size is reduced for incorporation of meat into the final product is the first step in the frankfurter process. This is done in a meat grinder, a bowl chopper, an emulsion mill, or a flaking machine. Grinders are usually employed as the first step in comminution of sausages. Emulsion mills are now the most commonly used for the preparation of the final comminution (Aberle et al., 2001). On a large scale grinding has been replaced with chopping which renders a fine emulsion. During the chopping process, particle size is reduced which encourages protein extraction. Simultaneously, all of the cure, phosphate, and one-third of the water or ice are added. The high salt concentration results in better protein extraction and is important for the formation of a stable emulsion. Protein extraction is increased with different cooking schedules; the end result should be a final internal temperature of 71˚C. This process may have antimicrobial activity due to the presence of phenols in the liquid smoke (Messina et al., 1988). Further, Gedela et al. (2006) found that liquid smoke

extract was effective in preventing the growth of *L. monocytogenes* for as long as 10 weeks when using either a 1 second dip time or a simple spray device. After smoking, frankfurters are precooked in a cooking vat or in a water spray. An internal temperature of 70-71˚C should be reached during this process, although 68˚C is the minimum end-point temperature. This cooking process allows three things to happen (1) coagulation of protein, (2) fixation of the color and (3) pasteurization of the product. Immediately after pre-cooking, frankfurters are chilled and are kept in a chill room, or held at -18˚C until used. Frankfurters that are produced with a cellulose casing must undergo an additional peeling step. For proper peeling moisture is required between the sausage and the casing. This can be accomplished by moving chilled frankfurters from a cold room to a slightly warmer one or by applying a fine water mist to the product. The peeler knife is considered a major source of contamination in frankfurter processing. Nerbrink and Borsh (1992) evaluated the extent of bacterial contamination at each separate production stage in the making of emulsion-type sausages. These researchers found considerable bacterial growth (greater than 7 Log CFU/g) on sausages that were collected after cold storage and on sausages that had passed through the entire process, also > 7 Log CFU/g. Further it was found that bacterial contamination levels were the lowest at the heat processing (< 5 Log CFU/g) and packaging stages (< 5 Log CFU/g). When comparing the bacterial contamination levels they found that the cold storage room was the critical point, as bacterial recontamination was the most substantial during this point.

Levine et al. (2001) found the 10 year, 1990-1999, prevalence rate of *L. monocytogenes* to be 3.56 percent in 6,820 samples of small-diameter cooked sausage (including frankfurters). However, in 2003, Wallace et al.(2003), found a prevalence rate

of only 543 *Listeria* monocytogenes positive samples out of 32,800 commercially prepared frankfurters (1.65 percent). This data suggests that the prevalence rate of *L. monocytogenes* in frankfurters is decreasing. This is likely due to the use of one of the alternative processing strategies as outlined in FSIS Directive 10,240.4 (2009).

Ready-To-Eat

The definition of ready-to-eat is: a meat or poultry product that is in a form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes. A consumer survey conducted by Porto et al. (2004) found that only 29 percent of the individuals surveyed consider frankfurters ready-to-eat. A RTE product is not required to bear a safe-handling instruction (as required for non-RTE products by 9 CFR § 317.2(l) (2006) and § 381.125(b) (2006)) or other labeling that directs that the product must be cooked or otherwise treated for safety, and can include frozen meat and poultry products 9 CFR § 430.1 (2006). According to this definition frankfurters are classified as a ready-to-eat food product, and as such they are a high risk food. The International Life Sciences Institute (ILSI) identified the following risk factors causing a food to be classified as posing a high risk of being associated with listeriosis:

1. Potential for contamination with *L. monocytogenes*
2. Support the growth of *L. monocytogenes* to high numbers
3. Ready-to-eat
4. Requires refrigeration

5. Stored for an extended period of time

ILSI used these risk factors to conclude that frankfurters would be classified as a high risk product. Foong and Dickson (2004) found that the rate of attachment of *Listeria monocytogenes* to be high, between 84-87 percent, in ready-to-eat meats (including frankfurters) within 5 minutes. Furthermore, these researchers concluded that these sessile cells would not be detached under normal processing conditions. This indicates a need for an additional food safety measure to control the attachment and survival of *L. monocytogenes* in ready-to-eat meats. Because of the possibility of contamination of frankfurters with *L. monocytogenes* the USDA recommends that frankfurters should always be reheated before eating and to use a food thermometer to make sure they reach 73.8°C or are steamy hot throughout (www.fsis.usda.gov/Fact_Sheets/Hot_Dogs/index.asp).

Consumer Awareness and Food Safety Practices

Contamination of foods can often be tracked back to post-processing contamination in food-plants. However, the contribution of retail, in-home or restaurant contamination to human foodborne listeriosis has not been clearly defined. Homes, in particular kitchens provide a variety of opportunities for recontamination (Bloomfield, 2003; Redmond and Griffith, 2003). As with food processing establishment, pathogens may become established in homes for prolonged periods (Reij and Den Aantrekker, 2004). However, few studies have addressed the fate of pathogens in kitchens and households in detail. Food can become contaminated at the retail and food service level

as well as in the home, if safe food handling practices are not followed, such as hand washing and avoiding cross-contamination of food products.

Storage conditions and practices can vary greatly in homes of consumers and can generally contribute to or reduce the risk of foodborne illness due to *Listeria monocytogenes*. Byrd-Bredbenner et al. (2006) conducted a survey of 18 to 26 year olds at a major university. Their survey was designed to audit the kitchens of those participating to identify problems and to develop recommendations for education efforts. Respondents were asked about kitchen safety including:

a. Temperatures
    i. Food thermometer available
    ii. Refrigerator temperature within recommended range
b. Cold food storage
    i. Items in refrigerator spaced to allow good air circulation
    ii. Raw meat/poultry stored below produce and other ready-to-eat foods
    iii. Raw meat/poultry stored in a leak-proof container
    iv. Raw meat/poultry past its sell-by-date

These researchers found that performance was the lowest on the temperature scale. In general, the mean refrigerator temperature was 6.1 ± 3.6°C (range, 0-16°C). While

freezer temperatures were found to be low enough to keep foods safe they were not low enough to preserve the quality of the foods. In the Quantitative Assessment of Relative Risk (Food and Drug Administration, 2003) it was demonstrated that the impact of temperature on the risk of listeriosis was significantly greater than the impact of time. Only seven percent of the homes in the survey reported having a refrigerator thermometer. Higher refrigerator temperatures promote growth of foodborne pathogens, which could lead to illness if foods are not cooked or reheated properly. If a consumer's refrigerator could be maintained at 5°C or lower, the incidence of listeriosis is estimated to be reduced by greater than 98% (Byrd-Bredbenner et al., 2006).

In a nation wide web-based survey of the U.S. population (18 year olds and older) Cates et al (2006) looked at consumer knowledge and handling practices of frankfurters and deli meats. Approximately 60 percent of the respondents reported at least one individual in the at risk population. Only 43.8 percent of respondents were aware of *Listeria*, compared with 94 percent reporting awareness of *Escherichia coli* and *Salmonella*. Furthermore, of the consumers aware of *Listeria*, 67.4 percent were unable to identify a food vehicle. Less than six percent correctly identified fruits and vegetables (5.4%), seafood (4.5%), cheese (3.8%), milk (3.6%) and processed meats (3.1%) as foods likely to be associated with the pathogen. Respondents with young children in the home were more aware of *Listeria* than those with an elderly person in the home.

Ninety-six percent of the households that stored frankfurters stored the unopened packages for the recommended time of ≤14 days. Of these, 87 percent stored the packages in their freezer after opening, stored them in the refrigerator for the

recommended time ≤ 7 days or discarded the uneaten product. These researchers reported that 99.8 percent of those surveyed heated frankfurters before consumption. Not surprisingly the findings of this survey suggest that storing foods outside the USDA recommended guidelines was lowest among households in which the respondents had some college or a bachelors degree or higher. While not shown to be significant (P=0.05) the survey suggests that there may be a relationship between age categories and the likelihood that frankfurters will be stored outside the USDA guidelines. Individuals that are 18-29 years old were more likely to store frankfurters outside the guidelines than individuals that are 60 years old and older. While this study did show that consumers are largely unaware of *Listeria*, the number who are aware has increased. A survey conducted in 1993 found that less than 10 percent of respondents had heard of *Listeria* and of these only one percent could correctly identify a food vehicle likely associated with the pathogen (Altekruse et al., 1996).

Manufacturers of food rely on a variety of preservation and processing methods to produce a safe, wholesome product with a suitable shelf-life and consumer acceptability. These methods ensure the foods safety by inactivating or inhibiting the growth of spoilage and pathogenic microorganisms, suppressing undesirable chemical and biochemical changes, as well as maintaining its desirable physical and sensory properties. Methods currently used in food preservation involve physical, chemical or biological factors. For the purposes of these studies chemical and physical methods will be examined. Physical preservation factors include heating, cooling, radiation, high-pressure processing and packaging. Chemical treatments include addition of antimicrobial agents, acidifying agents or curing agents.

OBJECTIVES

The objectives of these studies were to evaluate non-lactate formulated frankfurters for the effectiveness of sodium lactate and acidified calcium sulfate applied as a surface treatment prior to and after inoculation of *Listeria monocytogenes* and stored for 120 days at 4°C, and to determine the antimicrobial affects of sodium lactate and acidified calcium sulfate on frankfurters formulated with and without lactate in the original frankfurter formulation during a study mimicking home storage conditions.

The research hypothesis for these studies is that sodium lactate will control the outgrowth of *L. monocytogenes* more effectively than acidified calcium sulfate throughout the course of these studies, thus having greater antimicrobial properties.

MATERIALS AND METHODS

Inoculum Preparation

The inoculum preparations for these studies consisted of five strains of *Listeria monocytogenes*. *Listeria monocytogenes* strains were obtained from the culture collection at Kansas State University Food Safety and Security Laboratories. All strains were tested for purity using the Crystal Gram Positive test kit (BBL, Becton Dickinson, Sparks, MD).

The five strain cocktail for each study was prepared by growing each individual strain in 10 ml of Brain Heart Infusion (BHI) broth and incubated at 35˚C for 24h. The cultures were vortexed prior to combination. 5 ml aliquots of each *Listeria*

monocytogenes strain were added to 500 ml of 0.1% peptone water. The initial inoculum level ranged from 3.3 x 10^9 CFU/ml to 4.9 x 10^9 CFU/ml depending on the repetition.

Sodium Lactate

Sodium lactate was obtained from PURAC America of Lincolnshire, IL. The sodium lactate had an initial concentration of 80 percent (v/v), and for both experiments SL was diluted with sterile deionized water to a concentration of 12 percent (v/v), as per the manufacturer's directions. All of the SL used in these experiments was from the same batch.

Acidified Calcium Sulfate

Acidified calcium sulfate was also obtained from PURAC America of Lincolnshire, IL. ACS had an initial concentration of 36 percent (v/v), and for both of the experiments it was diluted with sterile deionized water to a final concentration of 12 percent (v/v) as per the manufacturer's directions. All of the ACS used in these experiments was from the same batch.

Frankfurters

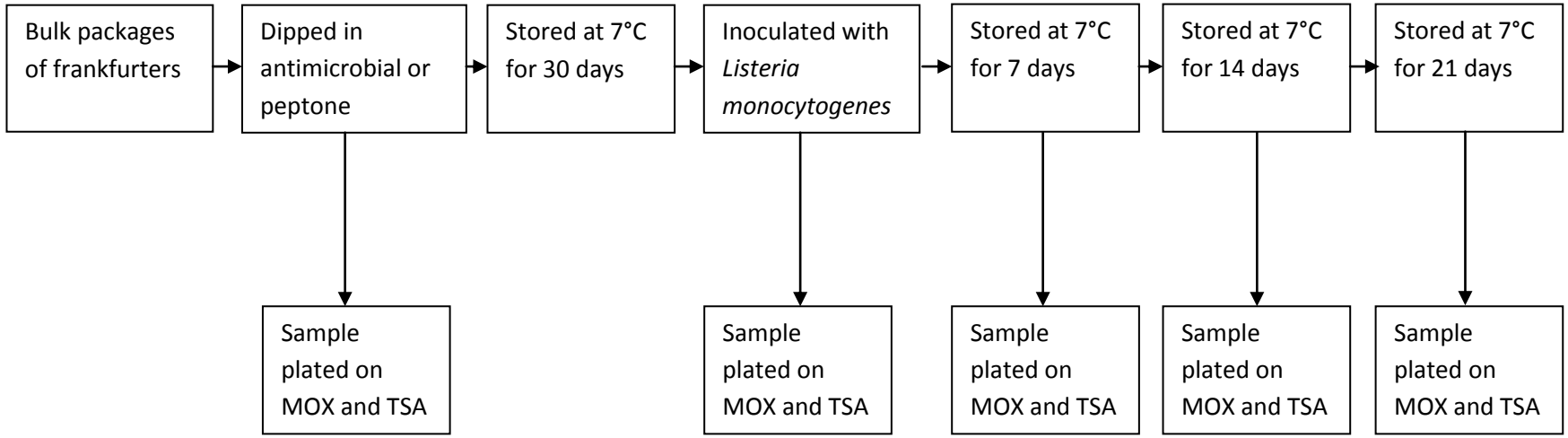
Frankfurters were supplied from Farmland (Wichita, Kansas). The frankfurters were jumbo links, fully cooked, skinless, and hickory smoked. The ingredients found in the frankfurters are as follows: mechanically separated chicken, pork, water, beef, salt, corn syrup, dextrose, flavorings, sodium phosphates, paprika, sugar, sodium erythorbate,

sodium nitrite; and were either made with sodium lactate or without sodium lactate in the formulation. Frankfurters arrived in bulk bags and were stored at refrigeration temperature (4°C) until used. Prior to each experiment, frankfurters were randomly assigned to a treatment and transferred to individual plastic storage bags or into vacuum packages.

Sample Preparation for the Study One

Five frankfurters were used for each of the controls and the treatments. Controls for this study were prepared by dipping frankfurters, with lactate and without lactate in the product formulation, in 0.1% peptone water for 10 minutes, stabilizing for 30 minutes, and then one frankfurter was immediately sampled. The other four frankfurters were vacuum packaged and placed into the refrigerator at 4 °C for 30 days. After 30 days the frankfurters were removed from the vacuum package and dip inoculated with a five strain cocktail of *Listeria monocytogenes* for 10 minutes. Frankfurters were stabilized for 30 minutes, and then one frankfurter was immediately sampled. Treatment one consisted of frankfurters that did contain SL in the product formulation and were dipped in 0.1% peptone water for 10 minutes, stabilized for 30 minutes, and then dipped in SL for 30 seconds. The procedure was repeated using frankfurters that did not contain SL in the product formulation. The other four frankfurters were vacuum packaged and placed into the refrigerator at 4 °C for 30 days. After 30 days the frankfurters were removed from the vacuum package and dip inoculated with a five strain cocktail of *L. monocytogenes* for 10 minutes. Frankfurters were stabilized for 30 minutes, and then one frankfurter was

Figure 4. Flow Diagram of Sampling Plan for Study One.



immediately sampled. Treatment two consisted of frankfurters without SL in the frankfurter formulation and were dipped in 0.1% peptone water for 10 minutes, stabilized for 30 minutes, and then dipped in acidified calcium sulfate for 30 seconds and were stabilized for 30 minutes, then frankfurter was immediately sampled. The other four frankfurters were vacuum packaged and placed into the refrigerator at 4 °C for 30 days. After 30 days the frankfurters were removed from the vacuum package and dip inoculated with a five strain cocktail of *L. monocytogenes* for 10 minutes. Frankfurters were stabilized for 30 minutes, and then one frankfurter was immediately sampled. This procedure was also performed with frankfurters that did not contain sodium lactate in the product formulation. For all treatments and the controls the three remaining frankfurters were individually placed into plastic storage bags at 7 °C and sampled after an additional 7, 14, and 21 days. Figure 4. shows the flow diagram of the sampling plan.

In addition to this experiment, the frankfurters formulation was evaluated to study the use of lactate in the formulation for microbial inhibition over a time. Five frankfurters in this treatment, that did not contain sodium lactate in the formulation, were dipped in 0.1% peptone water for 10 minutes, stabilized for 30 minutes, then one frankfurter was immediately sampled. The remaining frankfurters were individually vacuum packaged and placed into the refrigerator at 4 °C for 30 days. After 30 days the frankfurters were removed from the vacuum package and dipped in 0.1% peptone water for 10 minutes. The remaining four frankfurters were stabilized for 30 minutes, and then one frankfurter was immediately sampled. The three other frankfurters were placed into individual storage bags at 7 °C and sampled after an additional 7, 14, and 21 days. This

procedure was repeated using five frankfurters that did contain sodium lactate in the product formulation.

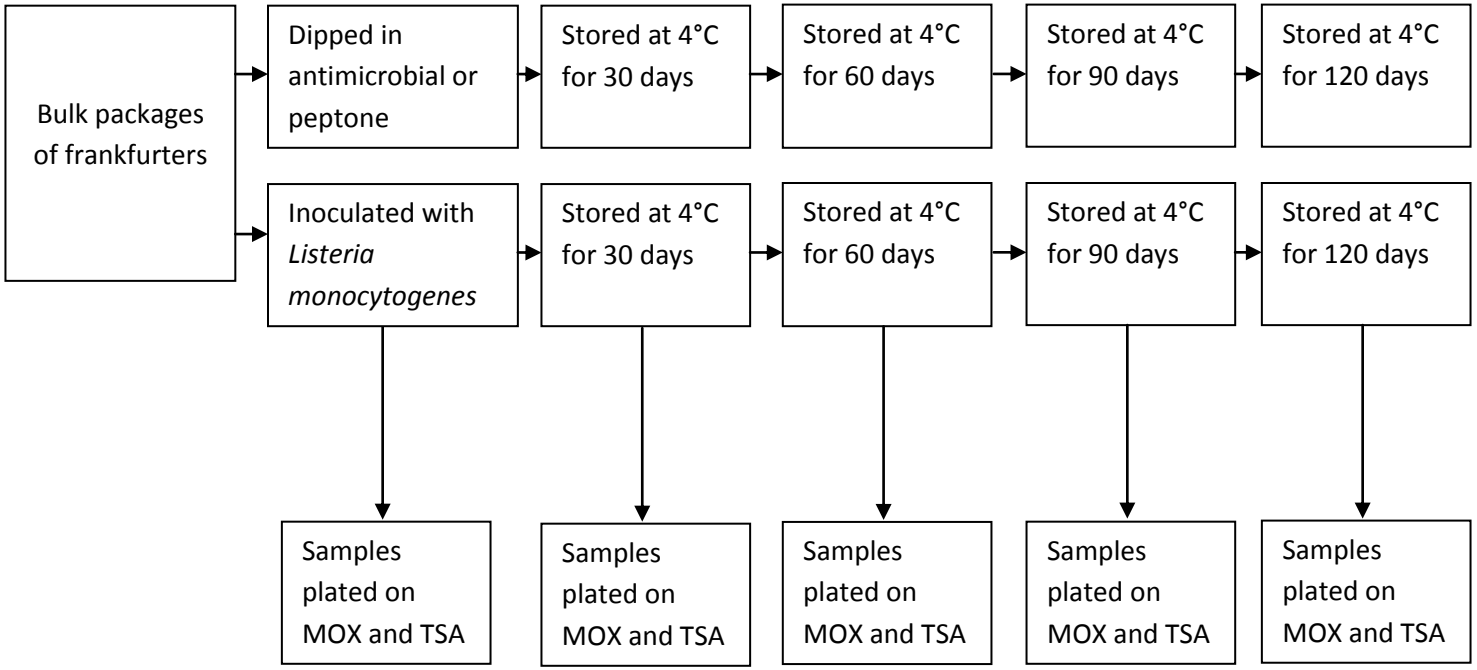
Sampling Methods

On sampling days frankfurters were aseptically transferred to filtered stomacher bags and 50 ml of 0.1% peptone were added. Frankfurters were pulsified using the Pulsifier (Microbiology International, Frederick, MD) for 1 min. Dilutions were made using 0.1% peptone water and plated on Modified Oxford Medium (MOX) (BBL, Becton Dickinson, Sparks, MD) and Tryptic Soy Agar (TSA) (BBL, Becton Dickinson, Sparks, MD) agar plates. MOX was used for isolation, enumeration, and differentiation of *Listeria monocytogenes* and TSA was used for bacterial aerobic total counts. The agar plates were then inverted and incubated at 35°C for 48h. Typical *L. monocytogenes* colonies, black with halos, were taken off of MOX plates randomly for each dilution at the end of the study and confirmed as *L. monocytogenes* using BBL Crystal Gram Positive test kits (BBL, Becton Dickinson, Sparks, MD).

Sample Preparation for Study Two

For each of the treatments five frankfurters were used. The control treatment consisted of frankfurters that were dip inoculated with a five strain cocktail of *Listeria monocytogenes* for 10 minutes and stabilized for 30 minutes in a biological hood. One frankfurter was sampled immediately. Treatment one consisted of frankfurters that were first dip inoculated for 10 minutes, stabilized for 30 minutes, and then dipped in sodium

Figure 5. Flow Diagram of the Sampling Plan for Study Two.



lactate for 30 seconds. One frankfurter was sampled immediately. Treatment two consisted of five frankfurters that were first inoculated with the *L. monocytogenes* cocktail for 10 minutes, stabilized in a biological hood for 30 minutes, and then dipped in acidified calcium sulfate, one frankfurter was sampled immediately. Treatment Three consisted of frankfurters that were dipped in SL for 30 seconds, stabilized for 30 minutes, and then dip inoculated with the *L. monocytogenes* cocktail for 10 minutes. One frankfurter was immediately sampled. Treatment four consisted of frankfurters that were dipped in acidified calcium sulfate for 30 seconds, stabilized for 30 minutes and dip inoculated with the five-strain *L. monocytogenes* cocktail. One frankfurter was immediately sampled. The remaining frankfurters from each treatment and the control were individually vacuum packaged, stored at 4˚C and sampled after an additional 30, 60, 90, and 120 days. Figure 5. shows the flow diagram of the sampling plan.

In addition to this study, 10 non-inoculated frankfurters, five of which contained lactate and five of which did not, were evaluated to see if there was an antimicrobial effect of inclusion of sodium lactate in the frankfurter formulation. The frankfurters in these two treatments were dipped in 0.1% peptone water for 10 minutes, stabilized for 30 minutes, then two frankfurters were sampled immediately; one containing sodium lactate in the formula and one without. The other eight frankfurters were vacuum packaged and placed into refrigerator at 4°C and sampled on day 30, 60, 90, and 120.

Sampling Methods

On sampling days frankfurters were aseptically transferred to filtered stomacher bags and 50 ml of 0.1% peptone were added. Frankfurters were pulsified using the

Pulsifier (Microbiology International, Frederick, MD) for 1 min. Dilutions were made using 0.1% peptone water and plated on Modified Oxford Medium (MOX) (BBL, Becton Dickinson, Sparks, MD) and Tryptic Soy Agar (TSA) (BBL, Becton Dickinson, Sparks, MD) agar plates. MOX was used for isolation, enumeration, and differentiation of *Listeria monocytogenes* and TSA was used for bacterial total counts. The agar plates were then inverted and incubated at 35°C for 48h. Typical *L. monocytogenes* colonies, black with halos, were taken off of MOX plates randomly for each dilution at the end of the study and confirmed as *L. monocytogenes* using BBL Crystal Gram Positive test kits (BBL, Becton Dickinson, Sparks, MD).

Statistical Analysis

Treatments for each experiment were replicated three times, frankfurters for each treatment and replication came from separate and individual packages; data from replicate samples plated in duplicate were analyzed using SAS (SAS Inst., Inc., Cary, NC). Data were analyzed as repeated measures over time; the model included fixed effects of; treatment, day, and treatment x day; or frankfurter type, day, and frankfurter type x day. Random effects consisted of replication. The covariance structure was compound symmetry. The α level was set at 0.05 to avoid committing a Type I error.

RESULTS AND DISCUSSION

Study One Results

Treatments using frankfurters without lactate in the original formulation showed dipping in peptone, sodium lactate (SL), or acidified calcium sulfate (ACS) allowed for *Listeria monocytogenes* to increase over time. However, frankfurters that did not contain lactate in the original formulation that were dipped in SL prior to inoculation resulted in the lowest total counts from day 30 through day 51. Frankfurters dipped in ACS had higher ($p \leq 0.0044$) total plate counts than the SL dipped frankfurters, but the control was not significantly different from the ACS dipped frankfurter ($p = 0.3911$). In comparing SL to ACS, dipping in SL resulted in lower ($p \leq 0.0004$) total counts than dipping in ACS by day 51.

Similar results were seen with the *L. monocytogenes* counts as SL dipped frankfurters without lactate in the original formula, had the lowest counts from day 37 through day 51 (Figure 7). In this comparison ACS resulted in higher microbial counts than either the SL treatment or the control. SL treated frankfurters resulted in a tendency ($p<0.001$) to have lower *L. monocytogenes* counts than those of the control. However, ACS treated frankfurters resulted in counts were higher than the control frankfurters ($p \leq 0.0351$) and the SL treated frankfurters ($p\leq 0.0013$).

These results indicate that sodium lactate had an inhibitory effect on inoculated microorganisms as the counts either stabilized or slightly decreased from day 30 through day 37. This inhibitory effect was not seen with acidified calcium sulfate in this application as the counts increased throughout the duration of this study.

Figure 6: Total counts (plated on TSA) of non-lactate frankfurters dipped with 0.1% peptone (Control NL), sodium lactate (SLNL), and acidified calcium sulfate (ACSNL) and inoculated with *Listeria monocytogenes* after 30 days.

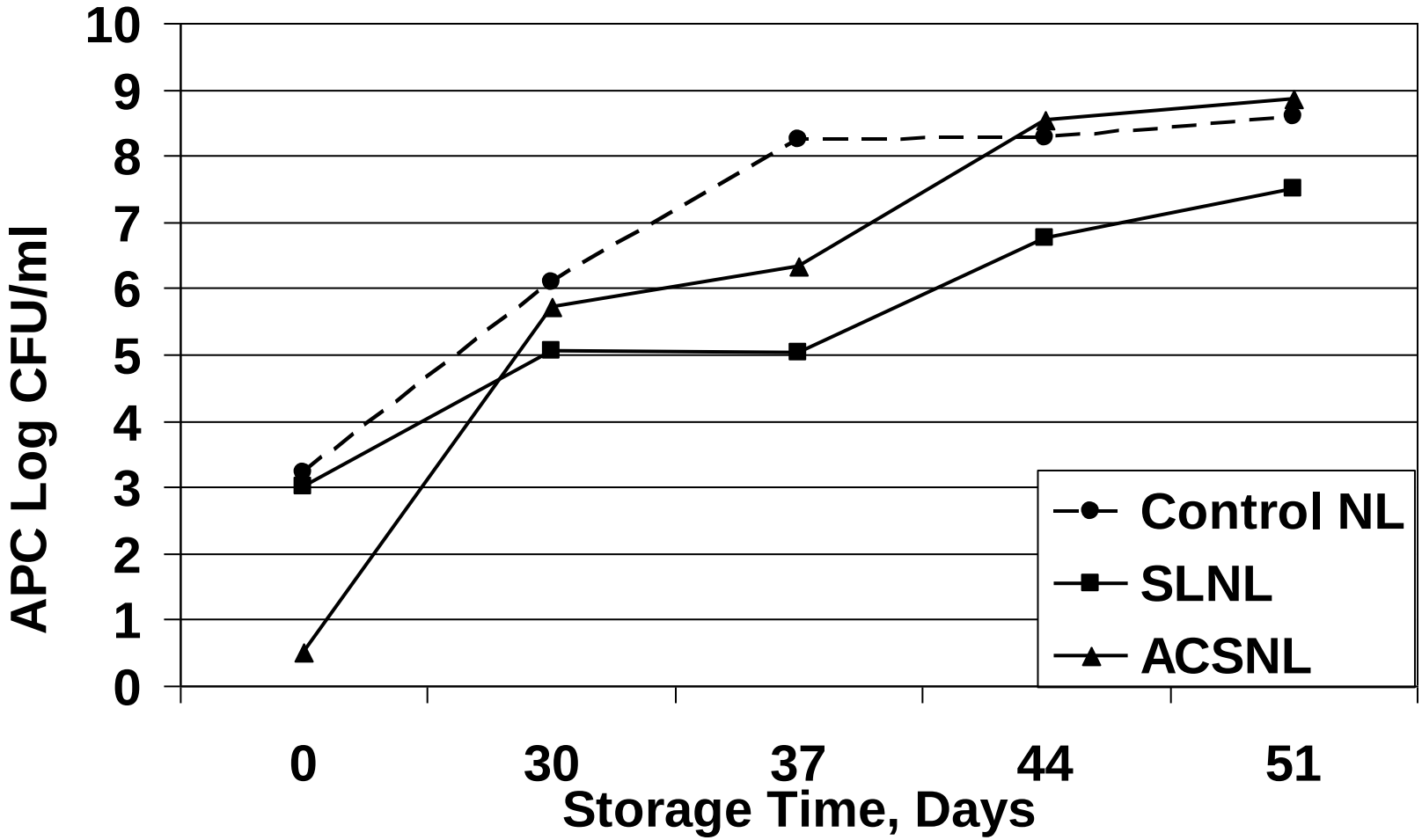
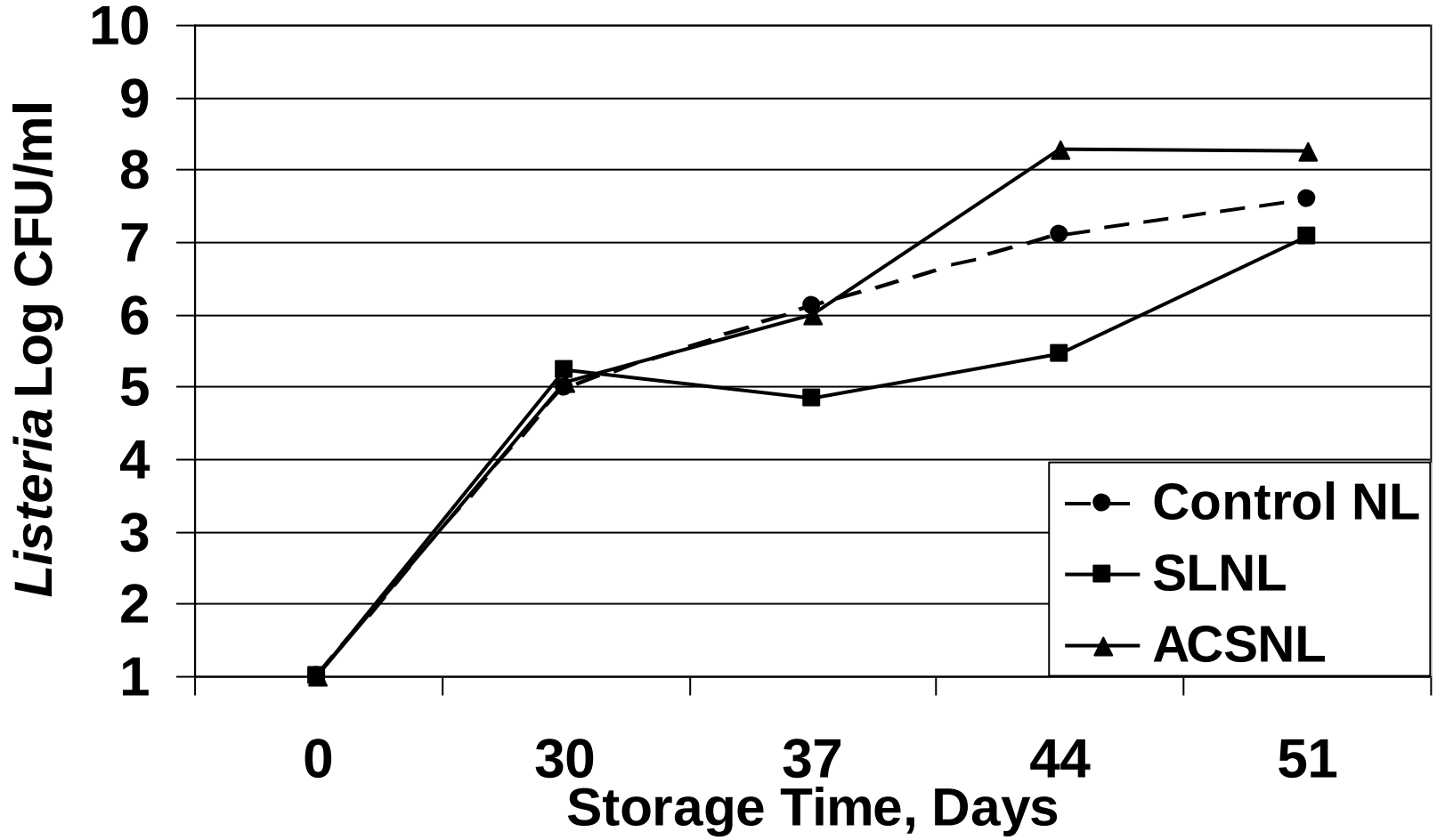


Figure 7: *Listeria monocytogenes* counts (plated on MOX agar) of non-lactate formulated frankfurters dipped with 0.1% peptone water (Control NL), sodium lactate (SLNL), and acidified calcium sulfate (ACSNL) and inoculated with *Listeria monocytogenes* after 30 days



Treatments using frankfurters with lactate in the original formulation showed that in the control treatments total counts increased over time. However, lactate-formulated frankfurters that were dipped in either sodium lactate or acidified calcium sulfate prior to inoculation 30 days later resulted in lower total counts from day 37 through day 51 ($p < .0001$).

The results were similar for the *L. monocytogenes* count comparisons between SL and ACS. Both dips resulted in lower counts than the control frankfurters by day 51 (SL, $p \leq 0.0005$; ACS, $p \leq 0.0026$) (Figure 7). In this comparison, dipping in SL did not result in significantly greater MOX count reduction than ACS.

The drastic increase in counts at day 30 was due to the inoculum addition. Over the next three weeks of storage both SL and ACS treated frankfurters showed a decrease in bacterial counts. This study showed that using lactate in the formulation of the frankfurters has a synergistic or multiple hurdle effect when dipping with either SL or ACS; as counts were much lower than frankfurters without lactate in the formula. Dipping lactate formulated frankfurters with either sodium lactate or acidified calcium sulfate was much more effective for *L. monocytogenes* reduction ($p \leq 0.0001$) than dipping frankfurters without lactate in the original formulation (Figure 10). Furthermore, Figure 10 clearly shows the difference between using lactate and non-lactate formulated frankfurters dipped with ACS.

Figure 8: Total counts (plated on TSA) of lactate frankfurters dipped with 0.1% peptone water (Control WL), sodium lactate (SLWL), and acidified calcium sulfate (ACSWL) and inoculated with *Listeria monocytogenes* after 30 days

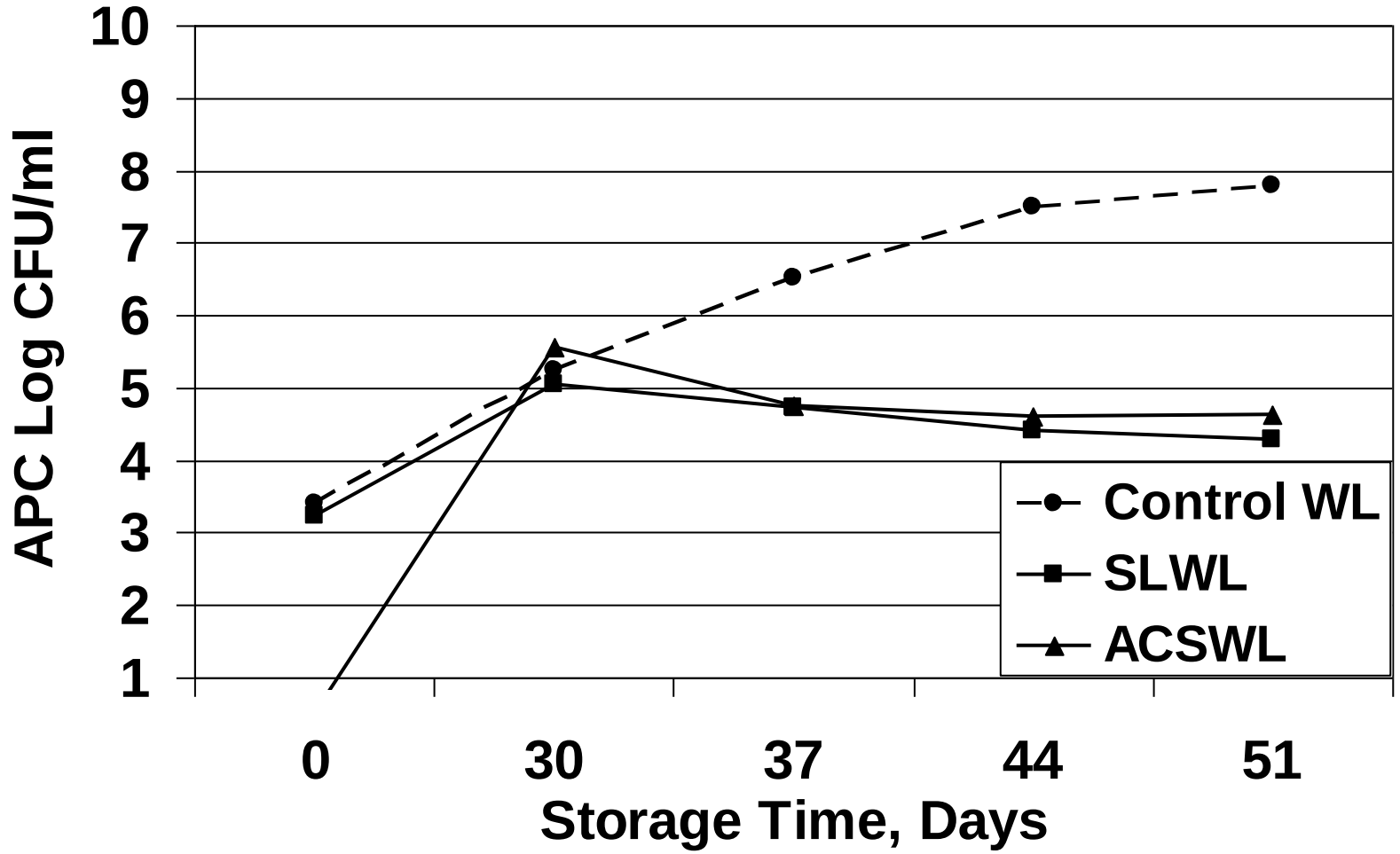


Figure 9: *Listeria monocytogenes* counts (plated on MOX agar) of lactate formulated frankfurters dipped with 0.1% peptone water (Control WL), sodium lactate (SLWL), and acidified calcium sulfate (ACSWL) and inoculated with *Listeria monocytogenes* after 30 days

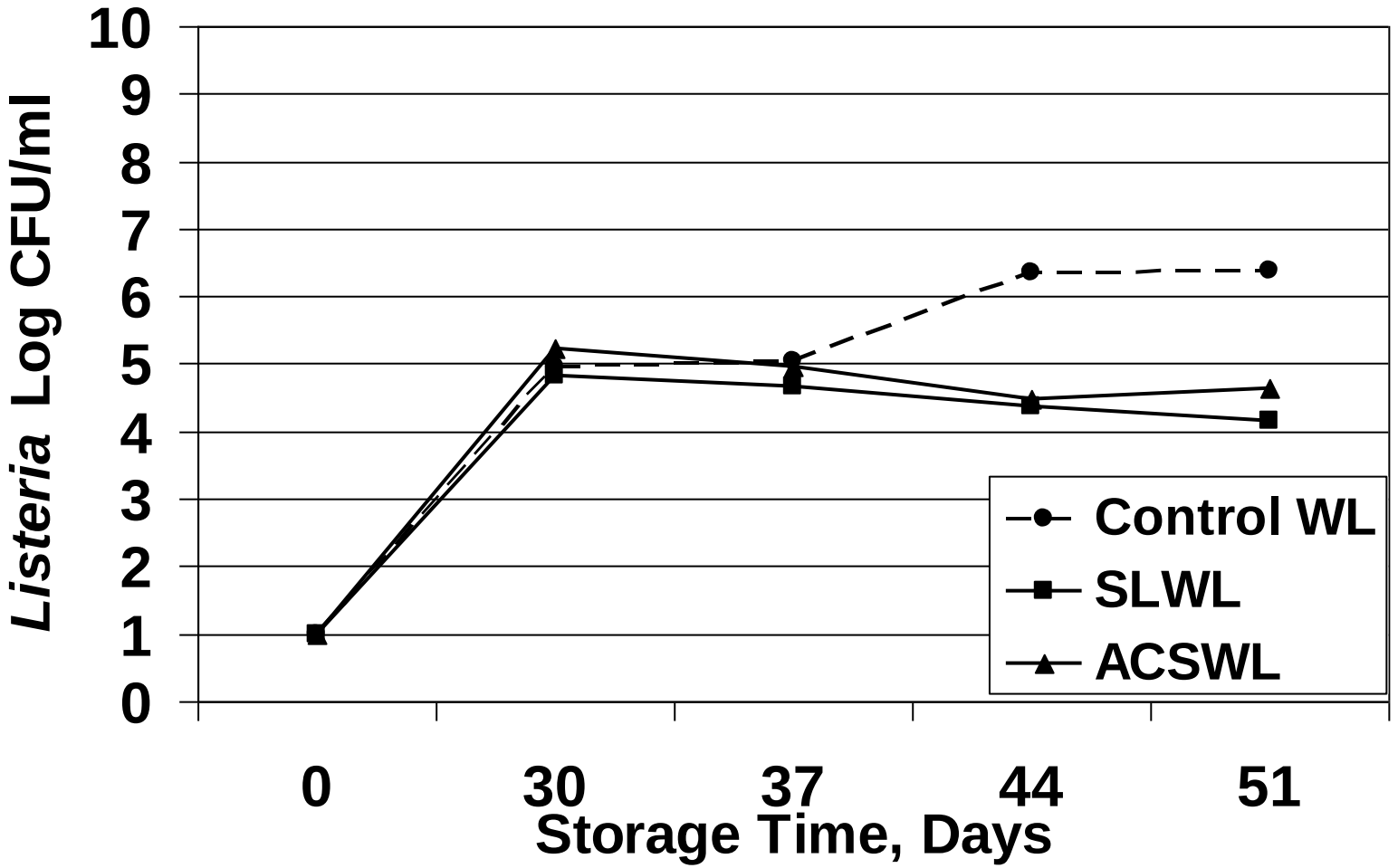
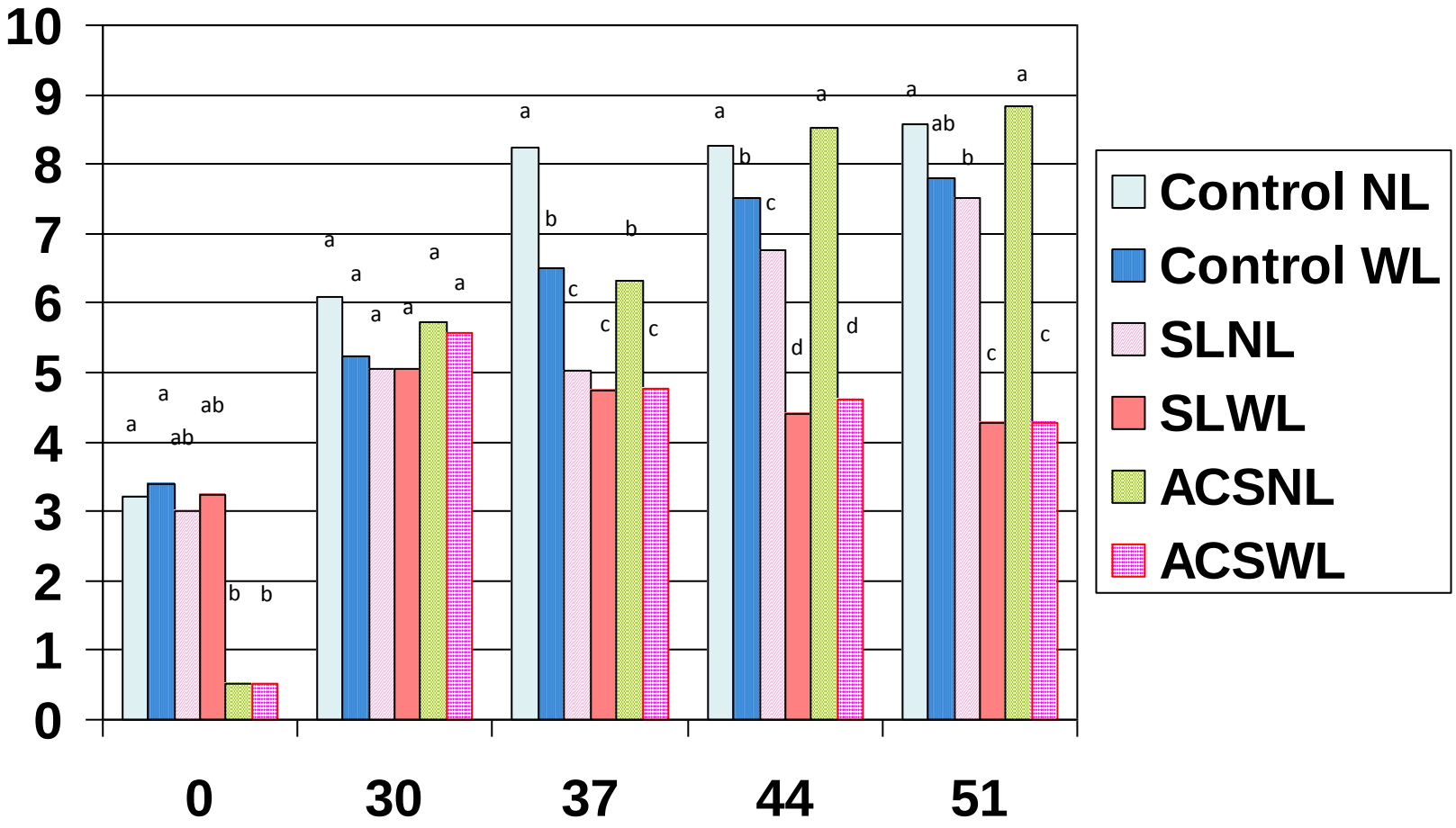


Figure 10: Tryptic Soy Agar (TSA) count comparisons of lactate (Control WL, SLWL, ACSWL) and non-lactate (Control NL, SLNL, ACSNL) formulated frankfurter. Columns within days with uncommon letters differ significantly ($p<0.05$).



Frankfurters containing lactate had lower counts than non-lactate containing frankfurters on day 30 ($p \leq 0.0013$), and day 37 ($p \leq 0.0157$) (Figure 11). The results of this research are similar to that of Samelis et al. (2002), who found that the inclusion of sodium lactate inhibited the growth of *Listeria monocytogenes.*

Study Two Results

These treatments evaluated the effectiveness of sodium lactate and acidified calcium sulfate for microbial reduction when the frankfurters were inoculated with a five-strain cocktail of *Listeria monocytogenes* prior to treatment with either SL or ACS. The results showed that after inoculation the total counts of the control frankfurters gradually increased 10^8 up to day 60, and then decreased to 10^7 CFU/ml by days 90 and 120. Total counts of the frankfurters that were dipped in either sodium lactate or acidified calcium sulfate were significantly lower than the control up to day 120 (Figure 12). Frankfurters that were dipped in ACS showed a steady increase in total counts from day 60 through day 120. In contrast to this, frankfurters that were dipped in SL did not steadily increase in total counts until day 90. In this study, the use of SL after inoculation with *L. monocytogenes* was more effective for microbial reduction than ACS. The frankfurters that were treated with SL after being inoculated had lower total counts on day 60 ($p \leq 0.0109$), day 90 ($p < 0.0001$), and day 120 ($p \leq 0.0043$). Furthermore, SL dipped frankfurters had significantly lower total counts than the control frankfurters throughout the entire study. Total counts of frankfurters that were dipped in ACS

Figure 11: Total count comparison of lactate formulated frankfurters (Lactate) with non-lactate lactate formulated frankfurters (Non-lactate) stored at 4°C for 51 days

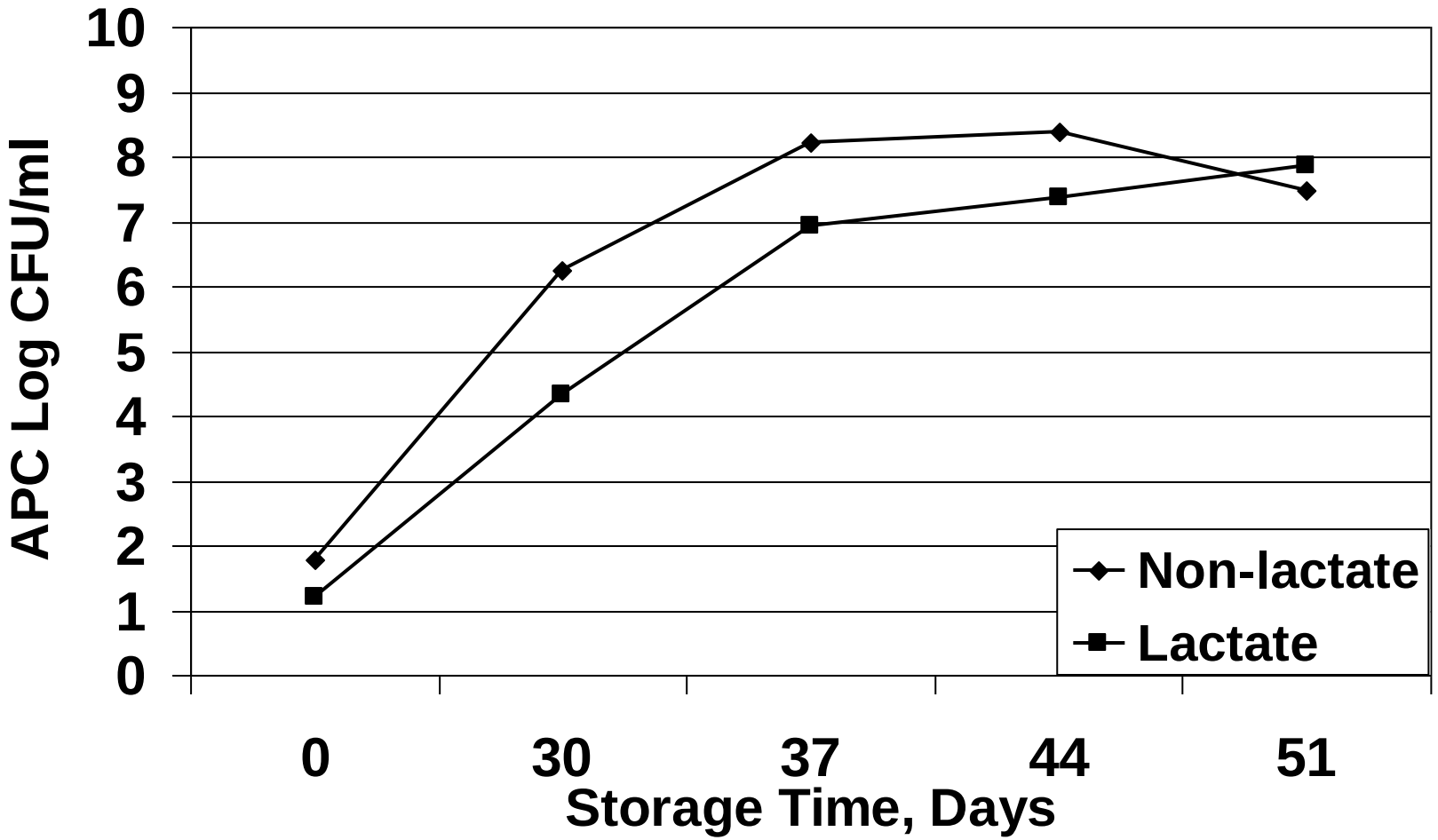


Figure 12: Total count comparisons (plated on TSA) of frankfurters inoculated with *Listeria monocytogenes* and then dipped in peptone water (Control) sodium lactate (SL) or acidified calcium sulfate (ACS)

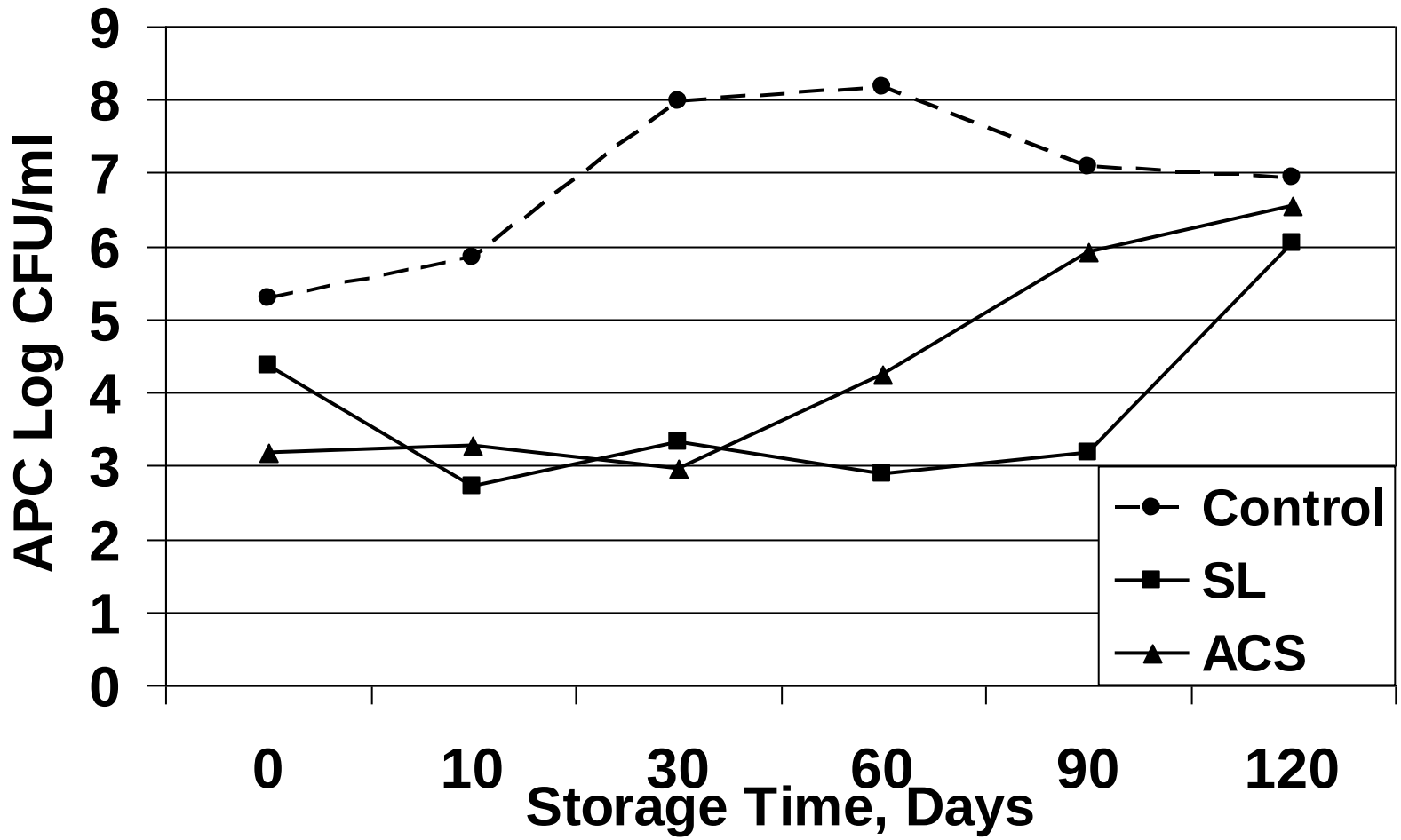
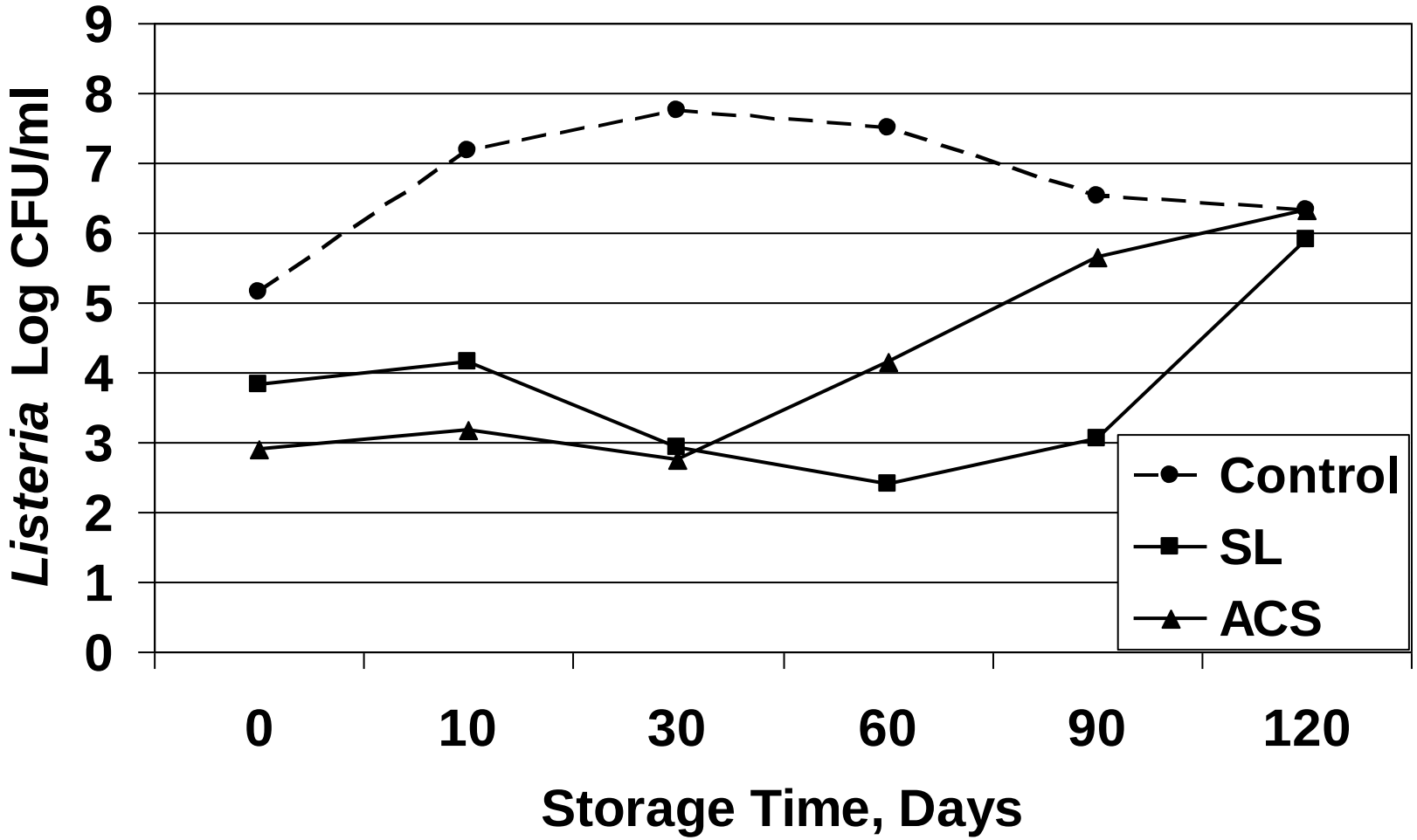


Figure 13: *Listeria monocytogenes* count comparisons (plated on MOX) of inoculated frankfurters (Control) with frankfurters inoculated and then dipped in either sodium lactate (SL) or acidified calcium sulfate (ACS)



increased from day 60, and were not significantly lower than the control frankfurters by day 120.

The comparison of *L. monocytogenes* counts (plated on MOX) was similar to the total counts (Figure 13). *L. monocytogenes* counts from frankfurters dipped in ACS were higher than the counts recovered from SL dipped frankfurters on day 60 ($p \leq 0.0052$) and day 90 ($p < 0.0001$).

These treatments evaluated the effectiveness of sodium lactate and acidified calcium sulfate for microbial reduction when frankfurters were dipped prior to inoculation with a five-strain cocktail of *Listeria monocytogenes*. SL treated frankfurter total counts were lower than the control frankfurter on day 30 ($p < .0001$, day 60 ($p \leq 0.0011$), and day 90 ($p \leq 0.0266$). Furthermore, SL counts were lower than ACS counts on day 30 ($p \leq 0.0027$) and day 60 ($p \leq 0.0004$). Total counts of frankfurters dipped with ACS prior to inoculation increased over time and were lower than the control only on day 30 ($p < .0001$).

The *L. monocytogenes* counts (plated on MOX) were similar to the total count results (plated on TSA) (Figure 15). Frankfurters dipped in SL resulted in lower counts than the control on day 10 ($p \leq 0.0285$), day 30 ($p < .0001$), day 60 ($p \leq 0.0001$), and day 90 ($p \leq 0.0521$). Also, it should be noted that *L. monocytogenes* counts from frankfurters treated with ACS were only 0.21 log CFU/ml lower than the control on day 90; and were actually 0.78 log CFU/ml higher than the control frankfurters on day 120. The results of this study contrast with that of Nunez et al. (2004), who found that surface application of acidified calcium sulfate had a bactericidal effect of *L. monocytogenes* throughout a 12

week study. The research conducted in this study showed that ACS does not have a bactericidal effect but rather a bacteriostatic effect. Sodium lactate was also shown to have bacteriostatic

Figure 14: Total Count Comparisons (plated on TSA) of inoculated frankfurters (Control) with frankfurters dipped in either sodium lactate (SL) or acidified calcium sulfate (ACS) and then inoculated with *Listeria monocytogenes*

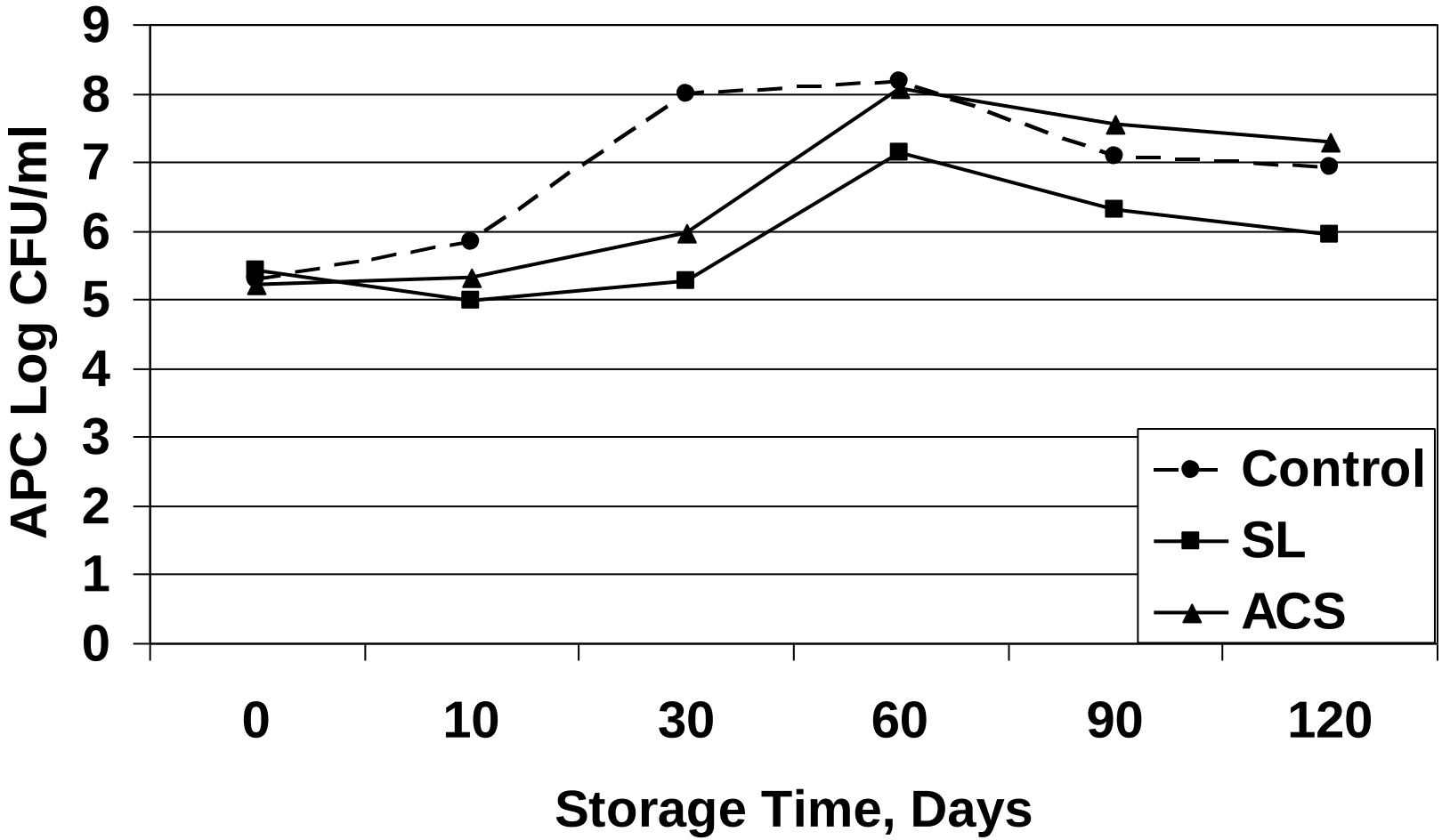
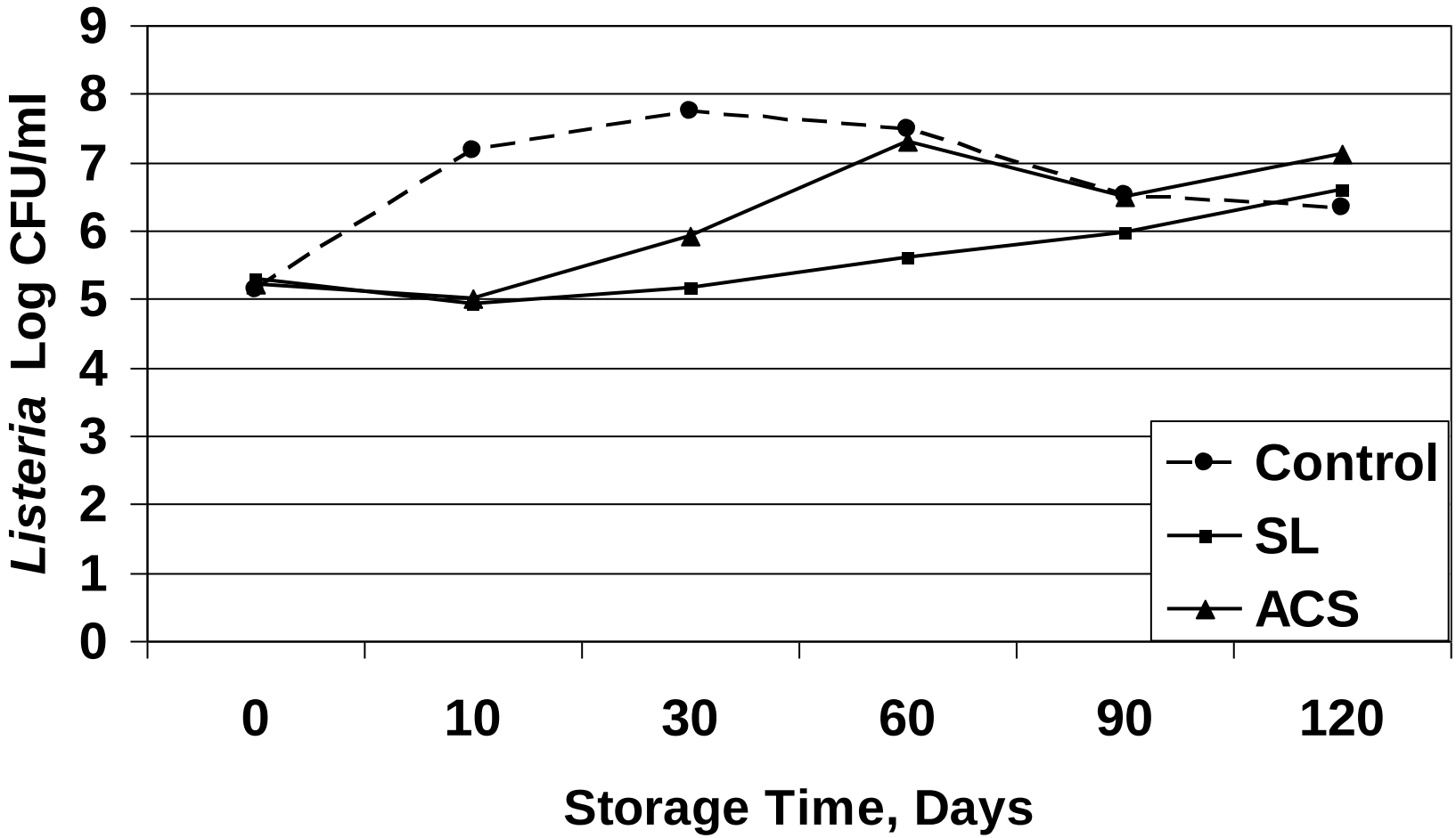


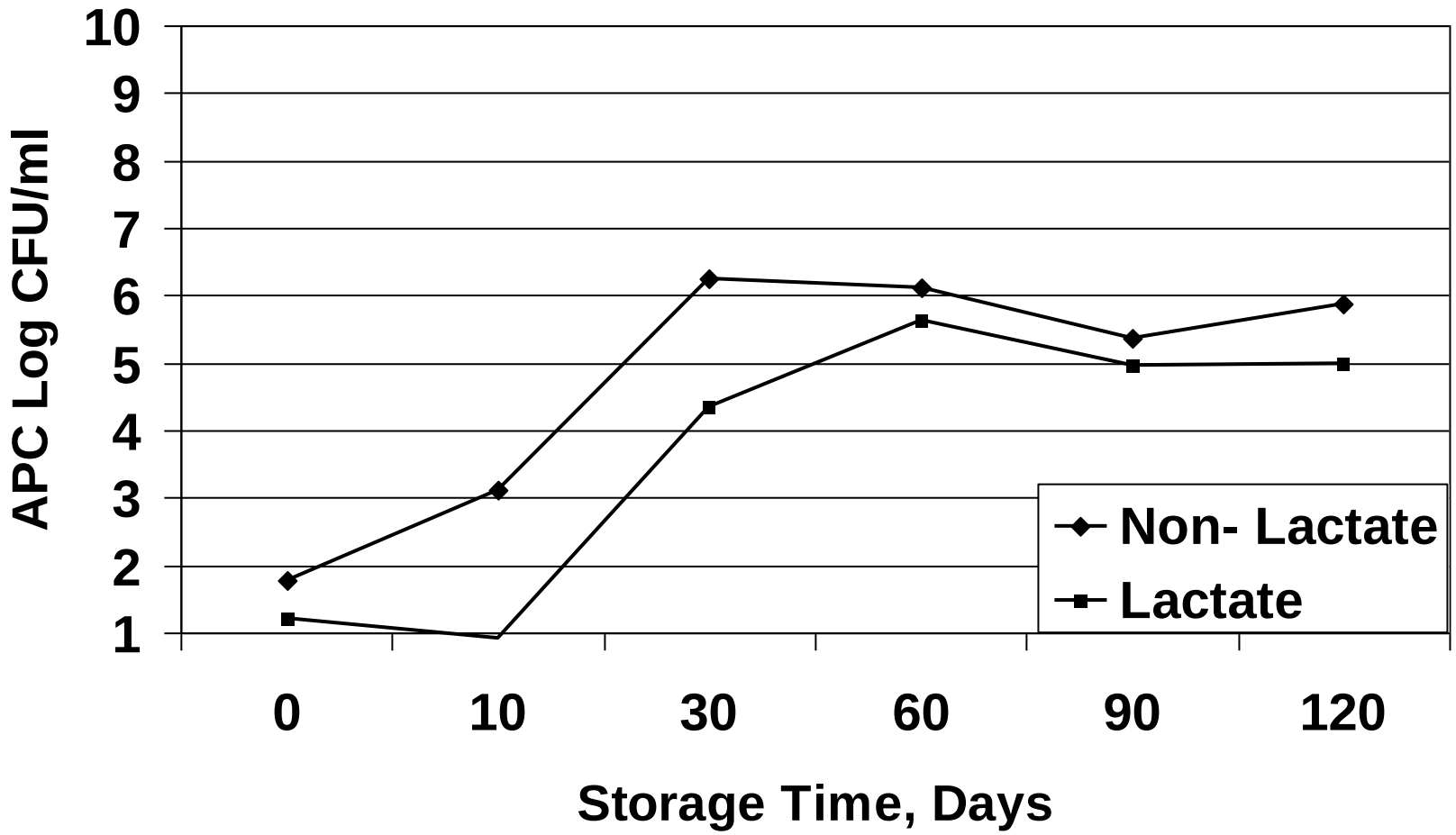
Figure 15: *Listeria monocytogenes* count comparisons (plated on MOX) of frankfurters either dipped in sodium lactate (SL) or acidified calcium sulfate (ACS) and then inoculated with *Listeria monocytogenes*



effects throughout the study. Figure 16 illustrates the microbial count differences between using sodium lactate in the original frankfurter formulation. Frankfurters containing sodium lactate had lower total counts throughout the study than the non-lactate containing frankfurters. These differences were significant on day 10 ($p \leq 0.0004$) and day 30 ($p \leq 0.0013$).

Figure 16 illustrates the differences in microbial load between frankfurters containing lactate in the original frankfurter formulation and frankfurters that were formulated without an antimicrobial in the formulation. Frankfurters that contained lactate had lower total counts throughout the study than the non-lactate containing frankfurters. These differences were significant on day 10 ($p \leq 0.0004$) and day 30 ($p \leq 0.0013$).

Figure 16: Total count comparison of lactate hot dogs (A1L) with non-lactate (A1N) hot dogs that were vacuum packaged, stored at 4 °C, and sampled at 0, 10, 30, 60, 90, and 120 days.



Summary

For the first study, an inhibitory effect was exhibited by non-lactate formulated frankfurters dipped in 12 percent sodium lactate; whereas this effect was not seen in frankfurters that were dipped in acidified calcium sulfate. Frankfurters formulated with lactate did show a synergistic effect when dipped in SL or in ACS in the home storage simulation study; although SL dipped frankfurters counts were lower. This same synergistic effect was shown by Samelis et al. (2002) when using sodium lactate in combination with 0.25 percent sodium acetate, sodium diacetate, or glucono-δ-lactine (GDL) in the product formulation. These researchers found that the combination of antimicrobials to be more effective for the control of *Listeria monocytogenes* than the inclusion of sodium lactate alone.

Lactate-formulated frankfurters that were inoculated and then dipped in SL showed the greatest reduction and were found to be most effective application. Overall, dipping the frankfurters prior to inoculation was not as effective at controlling bacterial growth as inoculating the frankfurters and dipping afterward, as total counts remained much higher throughout the study. Furthermore, less difference was seen between the control frankfurters and the frankfurters dipped in either antimicrobial prior to inoculation.

The inclusion of sodium lactate in the frankfurter formulation resulted in the lowest bacterial counts of both natural microflora and inoculated *L. monocytogenes* up to 120 days.

OVERALL CONCLUSIONS

Sodium lactate is effective as an antimicrobial dip on frankfurters for the reduction of natural microflora and inoculated *Listeria* monocytogenes up to 90 days. Using lactate in the formulation of the frankfurters has a synergistic or multiple hurdle effect when the frankfurter was dipped into either sodium lactate or acidified calcium sulfate. Dipping frankfurters prior to inoculation with *L. monocytogenes* was not as effective in controlling growth as inoculating the frankfurters and dipping afterward. This finding suggests that it may be of benefit to dip frankfurters in sodium lactate after peeling, to better control *L. monocytogenes* in frankfurters. In all studies, though not always statistically significant, sodium lactate was more effective at controlling outgrowth than was acidified calcium sulfate.

While the addition of both sodium lactate and acidified calcium sulfate were shown to have bacteriostatic effects throughout the study, neither compound had an inhibitory effect on *L. monocytogenes.* This is of importance because this research used levels of 12 percent which is much higher than the level that is used currently in the commercial manufacturing of frankfurters (4 percent). As was shown in this research and many others, the best method for the control of *L. monocytogenes* in frankfurters and other RTE foods may be by using combinations of antimicrobials. The synergistic effect of this may be further enhanced by inclusion of an antimicrobial in the product itself and then prior to packaging dipping in another antimicrobial, as was demonstrated in this research. Further, the inclusion of an antimicrobial into the packaging material or coating of packaging material along with addition of an antimicrobial into product formulation

(Marcos, 2007; Neetoo, 2007) may also help to control this pathogen. Recently, the FDA approved for the first time a bacteriophage to be used as a food additive for the control of *L. monocytogenes*. The additive is designed to be sprayed directly into meat and poultry products (www.fda.gov). The use of these bacteriophages in frankfurters alone or in combination with antimicrobials may also be more effective than using antimicrobials alone.

FUTURE WORK

The resulting data from these experiments shows that the use of single antimicrobials alone may not be sufficient to control the outgrowth of *Listeria monocytogenes.* In the future, work needs to be done to compare different combinations of antimicrobials for the reduction and control of *L. monocytogenes* and to find the optimum level and application of different antimicrobials that not only control outgrowth of this pathogen but also have consumer acceptability. Investigation of the use of antimicrobials in frankfurter formulations along with the use of dipping frankfurters in antimicrobials, and inclusion of antimicrobials into frankfurter packaging for the prevention of the outgrowth of this and other pathogens is needed. Comparing the control of *L. monocytogenes* by antimicrobial combinations to that of the newly approved bacteriophage (Food and Drug Administration, 2007) additive and investigation of the use of combinations of bacteriophage and antimicrobial is necessary for the control of *L. monocytogenes* in RTE foods.

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