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Survival of Listeria monocytogenes in a simulated recirculating brine chiller system

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Survival of *Listeria monocytogenes* in a simulated recirculating brine chiller system

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General Introduction

Listeria monocytogenes has relatively recently emerged as a foodborne pathogen of great importance. While only about 2500 cases of listeriosis occur each year, *L. monocytogenes* accounts for almost 30% of foodborne pathogen related deaths. Processed meats have been considered an important source of *L. monocytogenes* exposure since 1988.

The cost to the processed meats industry associated with *Listeria* outbreaks can be very high. Not only are the implications very serious for those who acquire listeriosis, but an outbreak can rapidly erode consumer confidence and trust in the contaminated product. Additionally, the cost of recalling product can be quite large. The 1999 outbreak at Bil Mar Foods cost the company over $75 million.

Most production methods for processed meats include a cooking step that is adequate to destroy more *L. monocytogenes* than could realistically contaminate the product. Therefore, post-cooking contamination becomes a very important issue for the processed meat industry.

One potential source of post processing contamination are recirculating brine chillers that are used to cool product immediately after cooking using a continuously recycled brine. Little work has been done on the ability of *L. monocytogenes* to survive in this system and the potential for contamination.

The purpose of this research was to evaluate the ability of *L. monocytogenes* to survive in these brine chillers at various pH and chlorine concentrations. This research included the use of a test tube system for preliminary work and then a model recirculating system for the final evaluation.

Literature Review

Introduction

Listeria monocytogenes is one of seven species found in the genus *Listeria*. Of the seven species, *Listeria monocytogenes* is the only one commonly associated with disease in man.

The morphology of *Listeria monocytogenes* is generally a short (~.5 X .5-2 μm), regular shaped rod or occasionally coccoid organism that occurs singly, in short chains or rarely in a filamentous form. The cells do not form spores and are classified as gram positive, not acid-fast organisms and are catalase positive. At the optimal growth temperature of 30-37 C the bacteria is not motile, although it does exhibit a tumbling motility when grown at 20-25 C. A distinguishing characteristic of *Listeria* is its ability to grow at temperatures near freezing. *Listeria* is classified as a facultative anaerobe meaning that it can survive and multiply in both the presence and absence of oxygen[1]. During medical isolation, *Listeria monocytogenes* can be easily confused with several other organisms. The palisade (many cells clumped side to side), Chinese letter like, or Y shaped formations of cells are often confused for *Corynebacterium*, a similar bacteria often implicated in diphtheria. Occasionally, *Listeria* may be rods over 10μm long in which case they are easily confused with *Erysipelothrix*, which also has similar characteristics and is associated with traumatic injuries and results in a painful, slow spreading, swelling of the skin. In addition, *Listeria* can often adopt a coccoid shape that can be confused with *Streptococcus*, a

common bacteria that cause a broad range of diseases including scarlet fever, meningitis and pneumonia. To further complicate matters, cells from older cultures and clinical samples often appear to be gram-negative[23]. Finally, *Listeria* is widely and ubiquitously distributed throughout nature.

History of *Listeria monocytogenes*

In 1924, Murray first characterized what is today known as *Listeria monocytogenes* from a culture obtained from laboratory rabbits[4]. The organism was named *Bacterium monocytogenes* because of the clinical monocytosis, destruction of monocytes, that it caused in rabbits. In 1925, an organism known as the "Tiger River Bacillus" was isolated from rodents in South Africa. The organism was officially named *Listerella hepatolytica* by Pirie out of respect to Lord Joseph Lister, the father of antisepsis[5]. In 1940 this organism became officially identified as *Listeria monocytogenes*[6]. It appears that *L. monocytogenes* was first isolated in 1919 as a "diphtheroid" from the cerobrospinal fluid of an ill soldier. However, this culture was maintained for over 20 years before it was identified as *L. monocytogenes*[7]. The first reported case of human listeriosis was isolated from a patient's blood sample by Nyfelt in 1929[8]. The first reported outbreak of listeriosis occurred in post World War II East Germany. *L. monocytogenes* was implicated in multiple cases of neonatal sepsis and meningitis. The outbreak was believed to have been caused by contaminated milk, although this was never conclusively shown. However, until the late 1970's listeriosis remained a medical oddity rather than an important illness[9].

Prior to the late 1970's, *Listeria* was more commonly viewed as a disease in animals. *L. monocytogenes* has been isolated in over 15 types of mammals and 20 species of birds, both domestic and wild, as well as frogs, fish, crustaceans, and ticks. Listeriosis in domestic animals was often attributed to consumption of poor quality silage which can have cell populations of >12,000 *L. monocytogenes* per gram[10]. Listeriosis most commonly occurs in sheep and cattle, with septicemia (infection of the blood stream), encephalitis (infection of the brain), and abortion being the most common symptoms. At this time, many of the documented cases of human listeriosis were in persons associated with the livestock industry[11].

During the period prior to 1979, epidemiologists were puzzled by very small sporadic outbreaks of listeriosis, especially small clusters of neonatal infections. Unfortunately, due to the small number of infected individuals in each outbreak, the vehicle of transmission was very difficult to discern. In frustration, investigators, at the Centers for Disease Control (CDC), referred to listeriosis as the "graveyard of epidemiology"[12]. The first clear evidence that listeriosis might be a food-borne infection came in 1979 during an investigation of an outbreak of nosocomial listeriosis in a city hospital in Boston Massachusetts. The investigators of this outbreak concluded that the source of a nosocomial infection involving 8 patients was most likely food served during their hospitalization[13].

Since the late 1970's, in addition to a low baseline of sporadic cases of listeriosis, there have been a large number of reported outbreaks (Table 1). Although all of these outbreaks play an important role in the history of *L.*

monocytogenes, there are three that not only provide insights into a classic outbreak, but have had a particularly large impact on the food industry.

In the summers of 1980 and 1981, an unusual number of listeriosis cases were documented in the maritime provinces of Canada. The theme of pregnancy being a predisposing factor for listeriosis was classically illustrated as 41 of 43 cases in this outbreak were perinatal. An epidemiological investigation into the source of the outbreak used a case-control study to conclude that consumption of a specific brand of coleslaw was significantly associated with listeriosis. More importantly, the epidemic strain of *L. monocytogenes* was discovered in coleslaw from one of the patient's refrigerators. This was a particularly important discovery as it was the first time that a specific food was not only linked epidemiologically, but also connected through microbiological investigation. Further research into the history of the coleslaw yielded another common characteristic of listeriosis outbreaks. The product had spent a prolonged (October to early spring) time in cold storage, an environment in which *L. monocytogenes* not only survives but can grow to large numbers. Finally, researchers also traced the cabbage back to a farm in which cabbage fields were fertilized with manure from a flock of sheep in which two animals had died from listeriosis. Thus, for the first time listeriosis was not only clearly connected with a food product, but the route of environment contamination could be plausibly theorized[14].

In 1985, another outbreak occurred that had a profound effect on the whole food industry, but more specifically on the dairy industry. The time following the outbreak became known as an era of " *Listeria* hysteria". The outbreak also fueled

an incredible amount of research on *L. monocytogenes* as is demonstrated by the flood of research articles published in the years following this outbreak.

During the first part of 1985, hospitals in Los Angeles County noticed an unusually number of listeriosis cases especially among pregnant Hispanic women. By the end of the outbreak, 93 perinatal and 49 non-perinatal cases were diagnosed, of which 48 had died (20 fetuses, 10 neonates and18 nonpregnant adults). A large investigation was launched and a soft Mexican cheese produced by Jalisco Cheese was implicated epidemiologically. A survey of the cheese products produced several packages that contained the epidemic strain and a physical inspection of the plant also yielded the epidemic strain. Further investigation demonstrated large levels of phosphatase still present in the milk, an indicator that the milk had not been properly pasteurized. Also plant receiving records indicated multiple instances in which the volume of milk received exceeded that pasteurization capacity of the facility[15 16].

This outbreak demonstrates another characteristic of many listeriosis outbreaks, that is, they are often connected with poor sanitation or process controls. As a result of this outbreak, the dairy industry, with some help from regulatory agencies, initiated sweeping changes in process controls, sanitation, and microbial awareness. Although this was a difficult time for the dairy industry, these efforts have increased the safety of milk products.

In late 1998 and early 1999, an outbreak of *L. monocytogenes* had a profound effect on the processed meats industry. Franks and deli meats produced by a Michigan processing plant, owned by Bil Mar Foods, were implicated in an

outbreak that covered 22 states, resulted in over 100 illnesses as well as 16 deaths and 6 miscarriages[17]. Over 35 million pounds of product were recalled at a cost of over $75 million. Although the cause of contamination was never conclusively determined, most investigators believe that it was a result of construction dust from another area of the plant. Whatever the source of contamination, this outbreak demonstrates what can happen if processed meats are contaminated after cooking.

Pathogenesis of *Listeria monocytogenes*

The pathogenesis of *L. monocytogenes* is quite different from most other foodborne pathogens. *L. monocytogenes* is considered an invasive intracellular pathogen. In fact, for many years, *L. monocytogenes* was used as the model for studying intracellular pathogens. In mice, within 10 minutes of intravenous injection, 90% of the pathogen has been captured by the liver, with the spleen capturing most of the rest. Most of the organisms are rapidly destroyed by macrophages known as Kupffer cells. However, any residual organisms then begin to grow in both the liver and the spleen and maximum levels are reached within 48-72 hours after logarithmic growth begins[18].

L. monocytogenes has a family of cell surface proteins known as internalins that prompt phagocytosis into epithelial cells and hepatocytes. Because the organism is phagocytosed by gastrointestinal cells, it doesn't disrupt the membrane while leaving the intestinal tract. Once engulfed by the cell, *L. monocytogenes* is enclosed in a phagolysosome which primarily functions to destroy invading bacteria. However, the low pH of this environment triggers production of listeriolysin O which

causes the phagolysosome to rupture releasing the pathogen into the cytoplasm of the cell. In this ideal environment, the organism replicates rapidly. *L. monocytogenes* then utilizes the actin machinery of the cell. Actin filaments normally provide structure and facilitate transport within the cell, but *L. monocytogenes* uses these filaments to push it into the adjoining cell. Thus the pathogen can spread rapidly without directly contacting the external environment. This life cycle seems to also explain why immunocompromised and pregnant individuals are more susceptible to listeriosis[19].

Because the organism is rarely in an external environment, cell mediated immunity seems to be the primary method of defense. In mice, the T cells attract blood monocytes to the sites of *Listeria* infection and then activate listeriocidal functions in macrophages that contain the organism. Murine listeriosis is generally short lived since once specific T cells are formed, *L. monocytogenes* is rapidly and efficiently destroyed[20]. Although the murine model is not a perfect human model, it does explain why only individuals with a weakened immune system are susceptible.

Clinical manifestations

Infection during pregnancy

Pregnant individuals are prone to listerial bacteremia. This is often exhibited as mild flu-like illness that generally occurs during the third trimester. Death among pregnant adults is very rare and the infection generally is resolved spontaneously following the end of the pregnancy.

Neonatal infection

Neonatal infection takes on either an early or late onset form. Early onset is often associated with pre-term labor and is acquired in utero. In utero infection often results in spontaneous abortion. Symptoms include meningitis, respiratory distress and heart failure. The overall mortality rate is > 50%. Late onset generally occurs ~2 weeks postpartum and is believed to be acquired during the birthing process. Infants with this type of infection exhibit irritability, poor feeding and meningeal irritation. Although the mortality rate is ~10% survivors may exhibit residual neurological damage.

Meningitis

Meningitis caused by *L. monocytogenes* is difficult to differentiate from other bacterial infections of the brain. This form of listeriosis is most common in newborns and males over 50. In individuals with underlying medical problems mortality rates can be as high as 70%, but in individuals with no underlying diseases, mortality ranges from 0-13%. Listeriosis is the fifth most common cause of meningitis, but has the highest associated mortality rate. Symptoms include movement disorders, seizures, fluctuating mental status and microabscesses of the brain.

Endocarditis

Infection of the heart lining is quite common and accounts for >7% of all cases of listeriosis. It is especially common in individuals with prosthetic valves and

often results in further medical complications. Mortality rate is nearly 50% and relapse is quite frequent. This infection often indicates the presence of other underlying medical conditions.

Cutaneous infection

Listeriosis can also be manifested as primary skin lesions. Illness is characterized by pin sized skin nodules that grow to pea sized with red edges and a pustular center. This infection generally spontaneously resolves. Although a rare manifestation, it is occasionally seen in farmers or veterinarians who come into contact with infected animal tissues, especially aborted fetal tissues or membranes.

Febrile gastroenteritis

In cases of a large infectious dose to healthy individuals, a foodborne illness similar to salmonellosis or campylobacteriosis can develop. Onset of fever, diarrhea, and vomiting are rapid (24-48 hours) and generally of short duration. Treatment is rarely necessary since the illness usually resolves spontaneously and further complications are rare.

Treatment

Without antibiotic treatment, invasive forms of listeriosis are most often fatal. All strains of *L. monocytogenes* are resistant to cephalosporins, which are the primary weapon used to fight meningitis. There are two commonly prescribed antibiotic courses. Traditionally, ampicillin and an aminoglycoside have been used

in combination, however recent treatment recommendations have included a combination of trimethoprim-sulfamethoxazole and rifampin. It is generally recommended that the antibiotics be taken for three weeks, although much longer treatments may be needed in immunocompromised individuals [5,9,18,21,22].

Detection of *Listeria monocytogenes*

The study of any bacteria requires the ability to select, isolate, and positively confirm its identity. Many of the "traditional" pathogens have one or perhaps two standard methods that have been proven over time to be highly effective. However, due to the rather recent and dramatic emergence of *L. monocytogenes*, a standard isolation method was not readily available and consequently a rather large number of methods emerged to meet the sudden demand for *Listeria* isolation. Isolation media and agars developed during the 1980's and early 1990's include McBride's *Listeria* Agar, Modified McBride's *Listeria* Agar, Martins Agar, Lithium Chloride-Phenylethanol-moxalactam agar, Modified Vogel Johnson agar, Modified Oxford Agar, Dominguez Rodriguez Isolation Agar, PALCAM, University of Vermont Broth, *Listeria* Enrichment Broth, Fraser Broth, and Universal Pre-enrichment broth[23]. Rather than describe each media in detail, I will describe some common selective ingredients, the original cold enrichment technique and the USDA isolation technique for meats.

Selective and Differential Agents

Temperature

The original selective agent for the isolation of *L. monocytogenes* is the use of cold temperatures. The ability of *L. monocytogenes* to grow at 4C has been exploited to provide a reliable, but slow, method for the detection of the pathogen. Storing the samples in a refrigerator for up to three months suppress other natural flora found in the sample while allowing *L. monocytogenes* to grow to detectable levels.

Potassium tellurite

This agent is an effective inhibitor of gram-negative organisms, but allows non-injured gram-positive *L. monocytogenes* to grow. It has been shown that this chemical inhibits repair in heat injured *L. monocytogenes*. Reduction of this additive by *Listeria* produces dark black colonies, aiding in the differentiation of the pathogen.

Phenyl ethanol and lithium chloride

These chemicals are also used to inhibit the growth of gram-negative organisms. They also appear to have inhibitory properties when the *L. monocytogenes* is in an injured state, especially heat injury.

Nalidixic acid

This antibiotic is also a selective agent for gram-positive organisms by inhibiting gram-negative bacteria and fungi. Research indicates that this supplement has little if any inhibitory affect on *L. monocytogenes*.

Acriflavin

This supplement is often used in combination with nalidixic acid. When used in combination, gram-negative and many gram-positive organisms are inhibited.

Moxalactam

Moxalactam is a cephalosporin class antibiotic. Research indicates that this antibiotic is inhibitory to almost all organisms except *Listeria*. This supplement is especially effective in samples that are highly contaminated[24].

Esculin and ferric ammonium citrate

These chemicals are not selective, but they aid in the differentiation of other organisms from the pathogen. *L. monocytogenes* has the ability to hydrolyze esculin. When this hydrolytic product reacts with ferric ammonium citrate a characteristic dark colored colony is produced.

Cold enrichment

This technique was originally implemented by Gray as a method for isolating pyschrophilic *Listeria* from environmental samples. The sample is placed in a nonspecific broth (such as nutrient broth), mixed well, and incubated at 4 C. At 1 and 4 weeks, the enrichment broth is streaked to a selective agar. The main disadvantage of this method is the long incubation time that is required to detect the pathogen. This becomes especially important in clinical cases, the detection of outbreaks, and in quality assurance situations at the production level[25].

USDA isolation technique for meats

This method was developed by the USDA for the detection of *L. monocytogenes* in meat products and is the method by which meat products are

evaluated for regulatory purposes. This method is similar to the FDA method for detection of *Listeria*, but it has been reported that the USDA method is more effective[26]. The sample is placed in University of Vermont broth (UVM), mixed and incubated at 30 C for 24 hours. UVM broth contains nalidixic acid and acriflavin which creates an effective selective media. Following incubation, 0.1ml is transferred to Fraser broth and a sample is also streaked onto Modified Oxford Agar (MOX) and both are incubated at 35 C for a minimum of 24 hours. Fraser broth contains lithium chloride, nalidixic acid, and acriflavin while the selective properties of MOX are attributable to esculin/ferric ammonium citrate, lithium chloride and moxalactam. After incubation, the Fraser broth is also streaked to MOX. Small dark colonies on MOX are considered presumptive positive for *Listeria*[27].

Further identification of *Listeria* Monocytogenes

Once a suspect colony has been identified, further tests are often conducted. First, it is necessary to determine if the colony is *L. monocytogenes* or one of the other 6 species of *Listeria*. Next, especially in epidemiological situations, a serotype and often genetic typing are conducted. Finally, in some situations, the actual pathogenicity of the organism is determined.

Determination of species

Of the seven known species of *Listeria*, only *L. monocytogenes* is commonly considered pathogenic. The other species include *ivanovii, innocua, welshimeri, seeligeri, murrayi*, and *grayi*. The species of *Listeria* is generally determined using a series of biochemical tests. These tests are summarized in Table 2[28].

The biochemical tests are often supplemented by the Christie-Atkins-Munch-Peterson (CAMP) test. Beta hemolytic *Staphylococcus aureus* and *Rhodococcus equi* are streaked vertically on opposite sides of a sheep blood agar plate. The suspect colonies are then streaked horizontally, without touching the other organisms and the plate is incubated for 24-48 hours at 35°C. Differentiation of species is determined by the zones of hemolysis in the area of the other organisms[29] (Table 3).

Serotyping and Genetic typing

Serotyping is the process of identifying an organism based on the presence or absence of certain extracellular (coat) proteins and flagellar proteins. The present system for *L. monocytogenes* serotyping differentiates 4 different serogroups and 19 serovars (1/2 a-c, 3 a-c, 4 a-e, 5, 6a-b) and is based on 14 somatic and 4 flagellar antigens. Of these serovars, 12 have been shown to cause disease, but 3 of these (1/2a,1/2b, 4b) characterize 95% of all human isolates. Unfortunately, because only three serovars are often associated with human disease[30], basing epidemiological studies on serotyping results can be difficult.

Molecular typing methods are much more useful to epidemiologists. The most common method used to differentiate strains of *L. monocytogenes* is a technique known as multilocus enzyme analysis. This technique identifies different strains based on characteristic patterns of metabolic enzymes. This technique had been able to divide the three main serovars into over 175 distinct strains. This has been very useful in the study of foodborne outbreaks and has allowed epidemiologist to pinpoint specific foods responsible for the spread of disease[31].

Pathogenicity testing

Although *L. monocytogenes* is the only species that regularly causes disease, it has been shown that not all *L. monocytogenes* strains cause disease. Additionally, growth conditions can also influence the pathogenicity of the organism. Therefore, the detection of *L. monocytogenes* in a food product does not necessarily constitute a health risk. Unfortunately, the only method for determining the pathogenicity of a particular strain is to use an animal model.

The animal model generally used to test the pathogenicity of *L. monocytogenes* is the immunocompromised mouse assay. In this test, five mice treated with carrageenan, to induce an immunocompromised state, are intraperitoneally injected with 10^4 CFU of the *L. monocytogenes* strain in question. If the mice do not die within three days, the *L. monocytogenes* sample is considered to be non-pathogenic[32].

As one might expect, the cost and time necessary to conduct this test tend to limit the usefulness of these assays. Due to these constraints, it is difficult to evaluate the differences in health risk within the *L. monocytogenes* species. As result, from a practical standpoint, any strain of *L. monocytogenes* detected in a food product is assumed to present a significant health risk[33].

Incidence and risk of *Listeria monocytogenes*

Following the Jalisco outbreak of 1985, there has been an increased interest in the incidence of *L. monocytogenes* in the United States. The majority of these studies show that the incidence of listeriosis is relatively low in the general

population, but significantly higher in pregnant, immunocompromised, elderly and newborn individuals. Additionally, they all show a rather elevated mortality rate and a sporadic nature attributed to multiple very small outbreak clusters.

In 1986, a group of researchers initiated a surveillance of ~14% of the U.S. population (from New Jersey, Missouri, Oklahoma, Tennessee, Washington, and California) for the incidence of *L. monocytogenes*. During the year long study, a total of 246 cases of listeriosis were detected for an overall incidence of .7/100,000 population, 12.7/100,000 live births (67 perinatal cases) and .5/100,000 population for nonperinatal individuals (179 nonperinatal cases). The mortality rate of perinatal individuals was 21% (no maternal deaths were reported) with the average age of the mother being 26, while the mortality among nonperinatal individuals was 35% and 84% of patients were over 50 years of age. When this model was applied to the whole United States, it was estimated that >1700 cases of listeriosis occurred, with ~450 adult deaths and ~100 fetal deaths occurring during 1986[34].

A retrospective summary of an outbreak period (December 1986-April 1987) that occurred in Philadelphia provided some interesting insights into the prevalences and mortalities during an outbreak. Thirty-six cases were identified during the study period for an overall incidence of 2/100,000 population, 4.4/100,000 population and 1.9/100,000 population for nonperinatal individuals. This is quite an increase considering the .3/100,000 population reported in the 11 months prior to the outbreak. The overall case-fatality rate was 44% and the mean age was 67 years[35].

A report of the incidences of listeriosis in selected areas of the United States (California, Georgia, Tennessee and Oklahoma) from November 1988 through

December 1990 presented by the CDC identified 301 cases. The annual incidence rate was .74/100,000 population with a mortality rate of 23%. One third of all cases were perinatal in nature and nearly all of the nonperinatal individuals had at least one underlying immunosuppressive condition[36].

In 1989 the USDA enacted a "zero tolerance" policy which effectively condemned any ready-to-eat foods found to contain *L. monocytogenes*. During this same time period, the food industry began a major effort to improve sanitation and reduce the incidence of *L. monocytogenes* in food. A follow-up study compared the levels of listeriosis in 1989 and 1993 in selected states throughout the country (Washington, California, Missouri, Oklahoma, Tennessee, Georgia, Maryland and New Jersey). The authors of this study felt that the actions implemented had effectively reduced the incidences of listeriosis. They found that perinatal listeriosis had declined from 17.4/100,00 population (<1 year old) in 1989 to 8.6/100,000 in 1993. In adults older than 50 years, the incidence had decreased from1.62/100,000 to 1.02/100,000. Overall, incidence dropped from .79/100,000 to .44/100,000. However, the death rate remained relatively constant with a 24% mortality in 1989 and a 22% mortality in 1993[37].

In 1999, the CDC released a landmark report on food-related illnesses and deaths in the United States. It was estimated that ~2500 cases of listeriosis occur each year. Most impressive was the fact that of an estimated 5 million cases of bacterial foodborne illness, *L. monocytogenes* accounted for almost 30% of the deaths. The 20% estimated case fatality rate is second only to *Vibrio vulnificus* among all foodborne pathogens[38].

A recent report by the CDC indicates that levels of listeriosis have remained fairly constant with an average incidence of ~.5/100,000 population. In the year 2000, 101 clinical cases of listeriosis were reported from a population of 29.5 million[39].

Information gathered in the above-mentioned and similar studies gives epidemiologist the opportunity to evaluate the risk posed by *L. monocytogenes.* The greatest difficulty in evaluating the risk posed by *L. monocytogenes* is that the infectious dose for immunocompromised individuals is unknown. Buchanan et al[40] developed a conservative dose-response relationship to help estimate the risk associated with foodborne *L. monocytogenes.* Using this relationship, Norrung[41] estimated that the risk of disease to an immunocompromised individual consuming 100g of smoked fish with 100cfu/g was less than 1 in 1,000,000. A separate health risk assessment of soft cheese for *L. monocytogenes* indicated that infectious dose for normal individuals was 10^7-10^9 while an infectious dose of 10^5-10^7 was estimated for high risk individuals[42]. Given these assessments and the ubiquitous presence of *L. monocytogenes* in low numbers, it is unlikely that low levels of the pathogen present a significant risk to immunocompromised individuals.

The USDA Economic Research Service (ERS) used the CDC data to determine that an estimated 2,298 hospitalizations and 499 deaths are caused by listeriosis. When the medical care and productivity loss associated with listeriosis are considered, the annual cost attributed to foodborne *Listeria* is $2.3 billion[43].

Presence of *Listeria monocytogenes* in the environment

As previously stated, *L. monocytogenes* is found ubiquitously throughout the environment. However, several studies provide a clearer picture of the distribution of the organism in the environment.

An informative study on environment prevalences was conducted in processing plants producing ice cream, cheese, milk, dry foods, potato products, frozen food as well as a sawmill and domestic environments. In the processing plants *Listeria* spp. was isolated from 15-53% of all samples taken except for the dry food plant were none was detected. *L. monocytogenes* was isolated in 5-20% of all samples taken. More telling was the fact that the majority of positive samples were recovered from floors, drains and standing water. In the sawmill, 8/109 samples were positive *Listeria* spp. with two of these being *L. monocytogenes*. Here again, most of the *Listeria* was isolated from damp areas. Interestingly, 20% of the 35 households studied were positive for *Listeria* spp. Five dishcloths from these houses were positive for *L. monocytogenes* and a positive sample was also isolated from a refrigerator[44].

In a larger study of household environments, similar result were recorded. *Listeria* spp. were detected in 47.4% of households and *L. monocytogenes* was detected in 21.1% of houses. The highest areas of contamination were dish cloths (*Listeria* spp. 37%, ~10^4 organisms/object) and bathroom sink drains (*Listeria* spp. 27.2%,~10^3 organisms/object). *Listeria* was also detected in areas such as kitchen sinks (5.7%) and refrigerators (3.9%). Here again it is evident that *Listeria* prefers a damp environment[45].

Prevalence of *Listeria monocytogenes* in food products

Since the recognition of food as the main source of *L. monocytogenes* much interest has been given to determining the amounts and prevalences of the organism on common food products. A 1989 study was not able to detect *L. monocytogenes* in 110 vegetable samples (lettuce, celery, tomatoes, and radishes). However, 56% (9/16) of chicken legs, 86% (38/44) of ground meats, 20% (6/30) of dry sausages, and a small number of ice cream samples (2/530) were positive for *L. monocytogenes*[46].

In a 1994 report, potatoes and radishes were characterized as the most consistently contaminated vegetables, most likely because of their close association with the soil. Interestingly, carrots have consistently been negative for *L. monocytogenes* and carrot juice appears to have an inhibitive effect in culture studies. In general, levels of the bacteria are generally <200 CFU/g[47].

A survey of prepacked retail sandwiches in Ireland was able to detect *Listeria* spp. in over 15% (113/725) of the samples. Five of the sandwiches had levels >100 CFU/g and two of these exceeded 1000 CFU/g. Chicken, beef, and bacon fillings were most often associated with a presence of the pathogen[48].

In a World Health Organization (WHO) report, it was reported that 6% of 18,000 foods were contaminated with *L. monocytogenes*, with 5% of these sample containing levels >1000 CFU/g. A summary of levels in dairy products reported 1-8% of raw milk samples and 1-10% of soft cheeses to be contaminated with the pathogens. Although generally low, levels in milk can reach 1000 CFU/g and 0.5-

5% of positive cheese samples had levels >1000 CFU/g. They also reported that 10-80% of ground and processed meat samples tested positive for *L. monocytogenes* although levels were <10-100 CFU/g approximately 90% of the time. Contamination in turkey products ranges from 11-20% at the processing plant while levels in chickens were 13-40%. Incidences as great as 66% in whole broilers, 28% of ready to eat grilled chicken, and 54% of frozen chicken have been reported. *L. monocytogenes* has also been isolated for 11-26% of fresh and cooked shrimp and 9-28% of smoked fish[49].

Of particular interest to the processed meat industry is a survey of *L. monocytogenes* in commercial franks. *L. monocytogenes* was isolated from 7.5% of 93 packages of franks consisting of 19 different brands. One particular brand, not included in the above 19, had a 71% incidence rate (17/24) for *L. monocytogenes.* Levels in this brand ranged from 4.3-27.6 CFU/ml[50].

World perspectives on *Listeria monocytogenes*

L. monocytogenes outbreaks in other countries, notably Canada and France, have created a worldwide concern about the control of this pathogen. While the U.S. has adopted a "zero tolerance" policy towards *L. monocytogenes*, other nations have adopted different strategies. In Europe, the tolerance limits for *L. monocytogenes* are determined by the product classification. Foods primarily for children must have a negative 25g sample while most other foods must have< 100 cfu/g or ml. The general attitude is that *L. monocytogenes* is impossible to eliminate from the environment and that foods are acceptably safe if they meet the <100 CFU

criteria[51], [52]. Australia has taken a similar approach by mandating a no-tolerance on products such as soft cheeses, processed meats, and smoked seafoods. Rather then imposing industry wide regulations, Australia has chosen to educate susceptible groups about risks and foods to avoid[53]. In Canada, only foods previously implicated in a recall or foods with a long shelf life that support growth of *L. monocytogenes* are subject to a no-tolerance policy. Other foods must meet a <100 cfu/g standard. Additionally, plant environment and end product are only sampled if a plant fails to meet sanitation and good manufacturing process requirements[54].

Listeria monocytogenes in ready to eat meats

Although *Listeria monocytogenes* was initially associated with dairy products and vegetables, its potential as a pathogen in ready-to-eat (RTE) meats (meats such as sandwich meats, hot dogs, and other meats in which cooking is not necessary prior to consumption) was quickly recognized. In a case-control analysis of selected listeriosis cases occurring during 1986 and 1987, cases (persons with the disease) were significantly more likely to have eaten uncooked (not heated by the consumer prior to consumption) hot dogs or undercooked chicken than the controls. In fact, 20% of the overall risk of listeriosis was attributable to consuming these foods[55].

In 1989, a case of listeriosis due to the consumption of turkey franks was identified and a subsequent evaluation of the processing plant by the CDC and USDA yielded some interesting facts. Environmental samples from areas prior to peeling and packaging of the franks, yielded very few positive samples, but nearly

90% of environmental samples of the peeling apparatus were positive for *L. monocytogenes*. Quantification of the levels of *L. monocytogenes* of the product purchased at a retail store indicated that these packages had less than 0.3 CFU/g, but samples taken from the patient's refrigerator contained >1100 CFU/g[56]. In general, contamination of RTE meats is a result of contamination at a single point in the production process following cooking, often by a relatively low number of organisms.

A 1994 study reviewed exposure factors of 165 listeriosis patients and 376 controls determined that foodborne transmission may account for a large number of sporadic listeriosis cases[57]. Upon examination of foods from the refrigerators of 123 of the listeriosis patients, 26 foods that matched the strain isolated from the patient were found. Analysis of the data led the researchers to concluded that RTE foods, contaminated with serotype 4b at levels of >100 CFU/g were strongly associated with the development of listeriosis[58].

A French study following up on an outbreak associated with foods bought at delicatessens determined that the major source of contamination was cross-contamination cooked and raw product via common food contact surfaces. In addition, they found that only a well-conducted and thorough sanitation program was able to eliminate *L. monocytogenes* from environmental sources[59].

A review of all the USDA recalls from 1994-2000 reveals that 136 different recalls were issued for meat products (Table 4). Notably, 46 of those recalls were for franks, 21 for sausage products, and 14 for both ham and lunch meats[60].

Clearly, to generate this many recalls, contamination by *L. monocytogenes* in ready-to-eat meats is a very large food safety concern.

Growth characteristics of *Listeria monocytogenes* in franks

Work by Zaika et al[61] showed that a normal cooking schedule reaching 160 F internal temperature was adequate to eliminate the 10^3 CFU/g or less of *L. monocytogenes* often found in raw ingredients for franks. The pathogen modeling program produced by the USDA, indicates that 0.75 minutes at 149°F is sufficient to obtain a three log reduction of *L. monocytogenes*[62]. Hence, adequately cooked franks should be free of *L. monocytogenes* prior to packaging. Other work has shown that waste fluids from clean-up of meat grinders can support growth of *L. monocytogenes* in areas such as drains with generation times as little as 2.3 hours at 8 C[63]. Clearly, some areas in a plant can encourage the growth of *L. monocytogenes* and so post-cooking environmental contamination becomes an important issue.

A study on the survival and growth of *L. monocytogenes* on Canadian retail franks vacuum packaged and stored at 5C for 28 days revealed that 40/61 samples supported growth of the pathogen. An average increase of 1.26 logs was recorded in the samples demonstrating growth[64]. Another study observed growth on ham, bologna, sliced chicken/turkey, franks and fresh bratwurst at 4.4 C and noted that products with a pH value >6.0 better supported the growth of *L. monocytogenes*[65]. An earlier study had also noted an increase from10^2 to 10^5 CFU/package in surface contaminated franks vacuumed packed and stored at 4C for 20 days[66]. This

research indicates that franks contaminated immediately prior to packaging and then stored for prolonged periods at refrigeration temperatures can harbor dangerous levels of *L. monocytogenes*.

Brine Chiller-A process that could contribute to post-cooking contamination?

In many frank production facilities, a process known as a brine chilling is often utilized to quickly chill the franks following cooking. Rapid cooling of the product improves product quality, yield, production time and reduces the time at which the product is at a favorable growth temperature for most pathogens.

The basic principle behind a brine chiller system is very simple. A 20% salt brine is cooled to ~-12 C. Following the end of a cook cycle, the chilled brine is sprayed from nozzles located in the top of the smoke house over the top of the cooked franks. The brine is then collected, cooled and then sprayed again. This process rapidly cools the franks, while conserving water and salt. However, heat and nutrients from the franks are transferred to the brine and there is some concern that psychrotrophic and halotrophic bacteria could become established in the brine chiller system and become a source of post-cooking contamination. The USDA has provided guidelines on recycled brine solutions, and has also recommended the addition of chlorine to the chiller system for sanitation purposes.

A study in simulated brine chiller conditions found that *L. monocytogenes* would not grow in salt concentrations >9%. However, at –12C and 20% NaCl, *L. monocytogenes* remained bacteriostatic (no growth, but little if any death) for the

entire trial period (30 days). Additionally, they found that lowering the temperature tended to have a protective effect on the pathogen[67].

However, as previously noted, the USDA recommends that chlorine be added as a sanitizer. Additionally, depending on the location of the processing plant, the pH value of the brine solution can vary between 6 and 8. Presently, no work has been done to evaluate the survival of *L. monocytogenes* in a brine chiller solution in the presence of chlorine and varying pH's.

Survival of *Listeria monocytogenes* in adverse conditions

While the survival of *L. monocytogenes* in a brine chiller with varying pH and chlorine levels can only determined experimentally, a large body of research exists about the reaction of the pathogen to conditions that exist in a brine chiller.

Salt

As has been previously mentioned, *L. monocytogenes* is quite tolerant to the presence of salt. Doyle summarized that *L. monocytogenes* has been shown to survive for 1 year in 16% NaCl at pH 6 and survived more than 100 days in 10.5-30% NaCl at 4 C[68]. Growth has been reported in media with NaCl concentrations as high as 12% in the temperature range of 8-30 C at pH 6.0[69]. Survival in high salt concentrations generally improves as the temperature decreases although at pH below 5 survival is better at 30 C than at 5 C[70].

When a microorganism is placed in a high salt environment, osmosis tends to draw moisture out of the organism into the environment. If this is not controlled, the cell would become shriveled and death would rapidly occur. The survival of *L.*

monocytogenes in high salt environments indicates that the organism has mechanisms that protect against destructive water loss. One mechanisms that has been identified in *L. monocytogenes* is the intracellular accumulation of compounds known as osmoyltes. The accumulation of these osmolytes counterbalances the forces of osmotic pressure and allows the organism to survive under harsh osmotic conditions. In addition, these osmoyltes are highly soluble, unreactive, and uncharged particles that do not adversely affect protein structure, enzyme-substrate interactions or protein-nucleic acid interactions. One of the most common osmolytes is glycine betaine which is common in small amounts in many organic food sources. This same substance also appears to provide protection to *L. monocytogenes* in cold environments[71]. Research has shown two primary methods for bringing glycine betaine into the cell. The first requires the presence of NaCl and takes advantage of Na+ gradient to transport the osmolyte into the cell. The second process uses an ATP-powered transport system to actively bring the glycine betaine into the cell[72]. While most of the information on the uptake of glycine betaine has been determined in vitro, other work has shown that *L. monocytogenes* rapidly accumulates glycine betaine and carnitine, another osmolyte, in meat systems using both processed and fresh meats. Additionally, uptake appears to be enhanced at lower temperatures[73].

Water activity

Water activity (a_w) is a measure of the amount of free water, or the water that bacteria or enzymes can utilize, in a food or system. In general, most important pathogenic and spoilage bacteria will not grow in foods with a water activity of less than .90[74]. Brief survival of *L. monocytogenes* in a 20% salt solution (a_w ~ .86.) has

been reported as well as extended survival for >1 year at pH 6 in a 16% salt solution (a_w ~.90)[75]. Growth of *L. monocytogenes* has been reported in media with an a_w of .92[76] with lower temperatures tending to increase the tolerance to the low a_w[77]. Additionally, in low pH conditions, a depressed a_w can have an antagonistic affect on the growth of *L. monocytogenes*[78].

pH

The pH or amount of acidity in a solution can have a dramatic effect on the survival and growth of *L. monocytogenes* in a liquid media. The minimum pH at which the pathogen can grow ranges from 5.0-5.7 depending on the acid used to achieve the pH[79]. *L. monocytogenes* has been shown to survive for over 90 days in orange serum with a pH of 4.8[80]. The effect of pH is also dependant on the temperature of the media with lower temperatures generally providing a protective effect[81 82 83].

Low temperature

The ability of *L. monocytogenes* to grow at low temperatures is a hallmark characteristic of the pathogen. Growth has been demonstrated at temperatures as low as –0.4 C in pasteurized milk with an accompanying lag phase of 3-4 days followed by generation times of ~80 hours[84]. More surprisingly, others have found although up to 82% of cultured organisms held for 14 days at –18 C were injured, 94-97% of the organisms were still viable[85]. When the ability of *L. monocytogenes* to survive in frozen foods was examined, the pathogen survived well in five different foods (ground beef, ground turkey, franks, canned corn, and ice cream

mix) with a pH of 5.8 or above, but populations greatly declined in a food (tomato soup) with a pH of 4.7[86].

The ability of *L. monocytogenes* to survive and even thrive at low temperatures is attributable to the osmolyte accumulation ability previously discussed as well as the composition of the cellular membrane. In non-psychrophilic bacteria, cold/freezing temperatures slow down enzyme reaction rates and interfere with transport across the cell member. Gelation, rigidity of membranes and cytoplasm due to freezing effect of low temperatures, results in membrane leakage, damage to DNA, and interruption/damage of RNA synthesis mechanisms[87]. *L. monocytogenes* avoids these problems by increasing the proportion of unsaturated fatty-acyl residues which increases membrane fluidity[88]. Additionally, *L. monocytogenes* grown at cold temperatures has more neutral fatty acids, which also aids in membrane fluidity[89]. It has also been shown that when the pathogen is suddenly exposed to cold temperatures at least 12 "cold shock proteins" are produced that appear to aid survival in this adverse condition[90].

Chlorine

Chlorine is one of the most popular sanitizers used in the food processing industry due to its effectiveness as well as relatively low cost. Chlorine does not have a specific cellular target, but rather, acts by denaturing many intracellular proteins and damaging protein synthesis functions. However, it doesn't appear that the cell wall is damaged[91]. When the effect of chlorine on *L. monocytogenes* is evaluated in a test tube system, the majority of cell destruction occurs in the first 30 seconds. Exposure to 0.5 ppm, 1ppm, 5ppm and 10ppm chlorine resulted in 0.5, 2,

6, and 6.5 log reduction respectively within one-half hour. However, the presence of organic material rapidly eliminated free chlorine from the solution[92]. Evaluation of phosphate buffer solution used to sanitize vegetables by dipping indicated that at least 50 ppm chlorine was necessary for destruction (~ 8 logs) of the pathogen. Interestingly, when inoculated brussels sprouts were dipped for 10 seconds in a 200 ppm chlorine solution, a destruction of only ~2 logs occurred[93]. Generally, *L. monocytogenes* attached to a surface rather than in a suspension require greater concentrations of chlorine as well as longer contact time (minimum of 5 minutes)[94] [95]. The effect of temperature on the activity of chlorine is not clear cut. It has been reported that greater concentrations of chlorine are needed at lower temperatures[96], cells are more susceptible to chlorine at lower temperatures[97], or that temperature has no effect on the efficacy of chlorine[98]. A reduction of pH appears to increase the efficiency of chlorine activity[97]. It has also been shown that growth of *L. monocytogenes* under low nutrient conditions increase resistance to chlorine[99].

Conclusion

This review of current literature has covered the importance of *Listeria monocytogenes* as a foodborne pathogen, medical symptoms associated, and an industrial process that needs to be evaluated for risk of *L. monocytogenes* contamination.

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Location	Date	Vehicle	No. of cases	No. of deaths	No. of perinatal cases	No. of non-perinatal cases
Halle/Germany	1966	Not known	279	-	-	-
Anjou/France	1976	Not known	162	-	134	28
Boston/USA	1979	Raw vegetables or milk?	20	-	-	-
Auckland/New Zealand	1980	Seafood?	21	-	-	-
Nova Scotia/Canada	1981	Coleslaw	41	17	34	7
Massachusetts/USA	1983	Milk?	49	14	7	42
California/USA	1985	Soft Mexican cheese	142	48	93	49
Vaud/Switzerland	1987	Soft cheese	122	31	63	59
Philadelphia/USA	1987	Not known	36	16	4	32
United Kingdom	1990	Pate	300	-	-	-
France	1992	Pork tongue in aspic	279	85	92	187
France	1993	Potted pork	39	-	31	8
France	1995	Soft cheese	36	-	18	18
USA	1998	Processed meat	38	4	6	32
France	1999	Pork tongue in aspic	26	8	-	-
USA	2000	Deli turkey meat	29	7	8	21
USA	2000	Soft Mexican cheese	12	5	11	1

Table 1. A summary of the most prominent historical *Listeria* outbreaks

Species	Hemolytic	Nitrate reduced	Mannitol	Rhamnose	Xylose	Methyl-mannoside
L. monocytogenes	+	-	-	+	-	+
L. ivanovii	+	-	-	-	+	-
L. seeligeri	+	-	-	-	+	Var.
L. innocua	-	-	-	Var.	-	+
L. welshimeri	-	-	-	Var.	+	+
L. grayi	-	Var.	+	Var.	-	+

Table 2. A summary of the metabolic profiles for the various *Listeria* species

Species	Staphylococcus aureus	Rhodoccus equi
L. monocytogenes	+*	-
L. ivanovii	-	+
L. innocua	-	-
L. welshimeri	-	-
L. seeligeri	+	-

* A (+) reaction indicates hemolysis

Table 3. Summary of the hemolytic patterns of the various *Listeria* species in the CAMP test

Product type	Number of recalls
Frankfurters	46
Sausage	21
Ham	14
Lunch Meats	14
Misc.	11
Chicken Salad	9
Cooked beef	7
Salami	6
Chicken	6
Pate	2
Total	136

Table 4. Summary of USDA FSIS recalls 1994-2000 divided into product type

Survival of *Listeria monocytogenes* in a Simulated Recirculating Brine Chiller System

A paper to be submitted to the Journal of Food Protection

J. K. Gailey, J.S. Dickson, and W. Dorsa

Abstract

Contamination by *Listeria monocytogenes* of processed meats following cooking presents a significant food safety risk. The purpose of this study was to determine the survival of *L. monocytogenes* in a simulated recirculating brine chiller system utilizing pHs of 5, 6, and 7 with free chlorine levels of 0, 3, 5, 10 ppm in a 20% salt brine at -12° C. At pH's of 5, 6, and 7 with chlorine levels of 2 and 3 ppm, using 10^8 CFUs in a test tube system, an immediate drop of 0.28 logs with no significance between treatments ($p>0.05$) followed by steady survival phase with a slope near zero was observed. In brine of pH 5 with 5 and 10 ppm chlorine an initial drop of 0.8 logs was observed followed by a steady survival phase with a destruction slope close to zero. At an inoculation level of 10^2 in a test tube system (pH 5 and 7 with zero and 10 ppm chlorine), the average initial drop for all treatments was 0.1 logs, followed by a steady survival phase. In a recirculating system, it was found that very few cells are destroyed during the brine chilling process, but that only low numbers of *L. monocytogenes* were recovered from the brine and uninoculated hot dogs. Although little destruction of *L. monocytogenes* was noted, the dilution effect observed during the study indicates that

environmental contamination of a brine chiller system poses little danger of post cooking contamination for processed meats.

Introduction

Listeria monocytogenes has been implicated in more foodborne illness related deaths than any other pathogen (8). Processed meats have been considered a common source of this pathogen since a case-control analysis released in 1988 linked consumption of uncooked (not heated by the consumer prior to consumption) hot dogs to an increased risk for listeriosis (12). Research by Zaika et al (15) showed that a normal cooking schedule reaching 160 F internal temperature was adequate to eliminate the 10^3 CFU/g or less of *L. monocytogenes* often found in raw ingredients for franks. Hence, adequately cooked franks should be free of *L. monocytogenes* prior to packaging. Other work has shown that waste fluids from clean-up of meat grinders can support growth of *L. monocytogenes* in areas such as drains with generation times as little as 2.3 hours at 8 C (4). Clearly, some areas in a plant can encourage the growth of *L. monocytogenes* and so post-cooking environmental contamination becomes an important issue.

In 1999, franks and deli meats produced by a Michigan processing plant, owned by Bil Mar Foods, was implicated in an outbreak that covered 22 states, resulted in over 100 illnesses as well as 16 deaths and 6 miscarriages (2). Over 35 million pounds of product were recalled at a cost of over $75 million (1). The USDA Economic Research Service (ERS) used the CDC data to determine that

an estimated 2,298 hospitalizations and 499 deaths are caused by listeriosis. When the medical care and productivity loss associated with listeriosis are considered, the annual cost attributed to foodborne *Listeria* is $2.3 billion (3). Clearly, the post-cooking contamination of processed meats can have a very large public health and economic impact.

Concern has been expressed that recycled brine commonly used to quickly cool processed meat products could serve as a source of post-cooking contamination.

The 1983 the FSIS issued a bulletin defining minimum salt concentrations and temperature conditions for recycled brine systems. In addition, the USDA has recommended that chlorine be added as an anti-microbial agent (13).

Little work has been done in evaluating this potential of brine chiller systems to act as a reservoir for *Listeria monocytogenes*. A study using a brain heart infusion broth system found that the pathogen grew at 5 C in 5% NaCl and at 12 C in 9% NaCl. Additionally, *Listeria monocytogenes* survived for 30 days at –12 C in a 20% salt solution (9).

In addition to variable salt, temperature, and chlorination conditions, the pH of water used in these systems varies between 6 and 8 at different geographical locations

The objective of this research was to evaluate the effect of both pH and free chlorine concentration, first in a test tube system, and then in a simulated brine chiller system.

Materials and Methods

Preparation of bacteria. A five strain cocktail of *Listeria monocytogenes* (Scott A, ½ a, and three 4b strains, all isolated from foodborne outbreaks) from frozen (-80 C) laboratory stock cultures (Food Safety Research Lab) were grown individually in Tryptic Soy Broth with 0.6% yeast extract (TSBYE) for 24 hours at 37 C and served as the stock culture for this study. From these cultures, 0.5ml of each strain was added to a single flask containing 100ml TSBYE. The broth was incubated at 20 C for 36 hours to simulate organisms from a plant environment. Thirty ml of this culture was centrifuged at 5000 X g for 10 minutes, after which, the supernatant was decanted and the cells resuspended in 30 ml of physiological saline solution (0.7% NaCl). After centrifuging again, the supernatant was removed and the pellet was resuspended in 3 ml of physiological saline solution. This solution was then serially diluted in 0.1% peptone water to achieve the desired concentration of cells and 0.1ml of the suspension was added to the brine.

Brine prepartion. The brine was prepared at a 20% salt concentration at pH's of 5, 6, and 7 by adding 200g of NaCl and 50 ml of an appropriate mix of a 0.1M citric acid/ 0.2M sodium phosphate dibasic buffer solution and bringing the volume to 1L with sterile distilled water. Tests on a recirculated brine chill solution found it contained approximately 0.1% fat and protein and so 0.1% lard (fat) and 0.1% bovine serum albumin (protein) were added to each liter of

solution. Due to the protein, the solution could not be autoclaved, rather plate counts were taken prior to inoculation of the brine and these counts were subtracted from experimental values.

Fifteen minutes prior to inoculation of the media, the appropriate amount of bleach was added to the brine to achieve the desired concentration of free chlorine. Free chlorine concentrations were determined using a Hach Free and Total Chlorine, 0-3mg/l, test kit (Hach Chemical Company, Ames, IA) in an uninoculated brine sample of the appropriate pH.

Brine models. Two different models were used in this study. The first model used triplicate test tubes containing 10 ml of brine. Following the addition of chlorine, the test tubes were allowed to achieve proper temperature by placing them in a commercial ethylene glycol-water mixture cooled to -12° C. using a cooling water bath (Fisher Scientific, Pittsburgh, PA).

Using the results from a test tube model, a simulated recirculating brine chill system was then evaluated. The system was set up using two separated 500ml erlenmeyer flasks brine reservoirs (one to inoculate and one to test) cooled to -12° C using the above mentioned water bath and a peristaltic pump and tubing to imitate the circulating action. Five hundred ml of brine was used in this experiment and again after the addition of chlorine, the brine was allowed to cool for 15 minutes prior to inoculation. In the first phase, the brine was contaminated and the effect of chlorine and pH (pH 5/10ppm chlorine and pH 7/0

ppm chlorine) in a brine system without franks over a period of four hours was evaluated.

In the second phase of the recirculation model, we evaluated the possibility of a contaminated hot dog spreading *Listeria* to other uncontaminated hot dogs via the brine at pH 5/10ppm chlorine and pH 7/0 ppm chlorine. The circulating system was set up the same as the previous experiment, except this time a contaminated hot dog was put in one reservoir and five uncontaminated hot dogs were placed in the other and the brine was circulated throughout the system (Figure 1). The pH and chlorine conditions tested in each model are shown in Table 1.

Meat preparation. Commercially produced hot dogs were purchased at a local retail outlet. The hot dogs were then cut into 5cm lengths, vacuum packaged in sets of 5 pieces, and irradiated at 7kgray to reduce populations of any natural flora.

The "contaminated" hot dog was inoculated by placing 0.5ml of previously described culture in the vacuum package and mixing by hand to ensure homogeneity.

Sample collection and bacteriological analysis. Brine samples were collected at appropriate time intervals by removing 0.1ml brine and serial diluting to appropriate levels in 0.1% peptone water+.0.03% sodium thiosulfate (to neutralize Cl^-). The samples were then plated on TSAYE using a WASP

automatic spiral plater (Don Whitley Scientific, Shipley, West Yorkshire, UK). In instances when *L. monocytogenes* levels were expected to be low, 0.25 ml was directly plated onto each of four separate plates using the spread plate technique to give a combined CFU/ml.

Hot dog samples were placed in sterile whirl-pack bags with 20ml of 0.1% peptone water+.0.03% sodium thiosulfate and the bags were agitated by hand for 60 seconds. The liquid was then filtered through an Iso-Grid .45μm hydrophobic grid and the grid placed on a TSAYE plate. All plates were incubated at 35C for 24 hours and counted using a ProtoCOL automatic plate counting system.

Data analysis. All data was analyzed using JMP statistical software. Mean values were determined for data with an apparent difference and these values were analyzed using a oneway Anova test for significant variance among treatments. The slope of data with no apparent difference was obtained using linear regression and this slope was tested to determine if it was significantly different from zero and a oneway Anova test for significant variance among treatments. Standard deviations and 95% confidence intervals were determined for data points near the detection limit.

Results

Test tube system. In the first phase of this experiment, *Listeria monocytogenes* counts were monitored for 15 minutes. The pH's 5,6, and 7 and free chlorine levels of 2 and 3 ppm were arbitrarily chosen since little data exists on the

subject. All pH's and chlorine levels exhibited an initial drop in plate counts that were not significantly different ($p>0.05$) with an average drop of 0.28 logs followed by steady survival. The slope of the loss of viable cells following this initial drop was significantly steeper (slope = -0.029) for pH 7, with 3 ppm chlorine ($p< 0.05$) when compared to all other treatments. The average slope of all lines combined was significantly less than zero ($p< 0.05$) with an average slope of –0.008 (Figure 2).

In the next phase, brine with a pH of 5 was evaluated at 5, 10, and 20 ppm free chlorine over 15 minutes. Brines with chlorine concentrations of 5 and 10 ppm were not significantly different from each other ($p> 0.05$) and exhibited an initial reduction of 0.8 log followed by a steady survival phase with a slope not significantly different from zero ($p> 0.05$). However, the brine with chlorine levels of 20 ppm eliminated *L. monocytogenes* to undetectable levels, a drop of greater 8 logs.

The final phase of the test tube system evaluated a more realistic inoculation level of 2 logs at pH 5 and 7 with no chlorine and 3ppm chlorine over 5 minutes. The treatment with pH 5 and no chlorine was significantly less than the other treatments ($p< 0.05$) with a reduction of –0.059. The average initial drop for all treatments was 0.1 logs, after which, the brine maintained a steady slope not significantly different from zero regardless of treatment ($p< 0.05$).

Circulating brine chill system. The first phase of the circulating system evaluated the effect of chlorine and pH in a brine system without franks over a

period of four hours. Two possibilities were evaluated, a control brine of pH 7, 0 ppm free chlorine and a brine of pH 5, 10 ppm free chlorine. There was no significant difference in the slope of the *L. monocytogenes* levels in either scenario throughout the course of the trial. The control brine maintained a level .30 lower levels than the low pH high chlorine brine, an artifact of the inoculation proceedure.

The second phase of the circulating system was similar to the first phase except that a contaminated hot dog ($2.2x10^3$-$5.4x10^3$ CFU/ hot dog) was used to inoculate the brine. The levels of *L. monocytogenes* on both the brine and uninoculated hot dogs were evaluated. Levels of *L. monocytogenes* isolated from both the treatment and control brines averaged 2.25 CFUs for brine and 0.33 CFUs for hot dogs. These levels were very near the threshold of detection and comparison of the 95% confidence intervals indicate that there is no difference between the two treatments. The most *L. monocytogenes* recovered from the brine was 10 CFU/ml and from the hot dog was 2 CFU/hot dog piece. Additionally, when the results of both brines are combined, contamination was sporadic and only 7/24 hot dog pieces were contaminated.

Discussion

The data obtained in the test tube system indicates that a brine of pH 7 with 3 ppm chlorine would reduce levels of *L. monocytogenes* more quickly than any other treatment. Also, at lower inoculation levels, pH 5 with no chlorine exhibited less of an initial drop than the other treatments. However, although

these findings were statistically different, these differences may not be large enough to be biologically meaningful in this model system. Additionally, it was found that 20 ppm chlorine effectively reduces *L. monocytogenes* to undetectable levels, while lower concentrations do not appear to be lethal to the pathogen.

The data obtained in the circulating system indicate that very few cells are lost during the brine chilling process. However, the low numbers of *L. monocytogenes*, after introduction via a contaminated hot dog, recovered both in the brine and on other hot dogs indicates that there is a large dilution effect.

The ability of *L. monocytogenes* to survive in a 20% salt brine at –12 C and low pH in the presence of free chlorine is quite remarkable. However, these results match quite well with existing data. As has been previously mentioned, *L. monocytogenes* is quite tolerant to the presence of salt. The ability of *L. monocytogenes* to grow at low temperatures is a hallmark characteristic of the pathogen. It has been found that even after being held for 14 days at –18 C, 94-97% of a pure culture of *L. monocytogenes* was still viable (7). Additionally, it has been reported that *L. monocytogenes* can survive for over 90 days in orange serum with a pH of 4.8 (10). The effectiveness of using chlorine as a sanitizer in systems containing high levels of organic materials has also been questioned (6). Evaluation of phosphate buffer solution used to sanitizing vegetables by dipping indicated that at least 50 ppm chlorine was necessary to for destruction (~ 8 logs) of the pathogen. Interestingly, when inoculated Brussels sprouts were dipped for 10 seconds in a 200 ppm chlorine solution, a destruction of only ~2 logs occurred (5).

While the test tube system provided valuable information about the reaction of *L. monocytogenes* to salt brine systems of various pH and chlorine levels, the circulating system provides valuable information about the hazard posed by *L. monocytogenes* in a brine chiller system. While it is clear that *Listeria* can rapidly disseminate through a brine chiller system and contaminate both brine and hotdogs, the dilution effect also becomes readily apparent. When the brine was contaminated with ~10^3 organisms, a feasible natural contamination, the levels in the brine were generally < 5 CFU/ml. Additionally, in our 500ml system, only 30% of the hot dogs were contaminated. When the same amount of contamination is introduced to a real system that contains >1500 liters of brine the dilution effect adds a margin of safety. It is unlikely that a natural contamination of 1x10^5, the amount needed to achieve a contamination of 1 CFU/ml in1500 L of brine, would occur. Additionally, when one considers the average 0.8 log reduction and the very few organisms that actually attached to the hotdogs during circulation, the chances of a single hot dog becoming contaminated is quite small. Finally, a *Listeria* testing program with more than 450 samples of two plant's brine chillers conducted for over a year failed to yield even one positive sample (Personal communication, Warren Dorsa).

Growth of *L. monocytogenes* has been demonstrated at temperatures as low as –0.4 C in pasteurized milk (14) and in media with NaCl concentrations as high as 12% in the temperature range of 8-30 C at pH 6.0 (11). However, the temperature (-12C) and salt concentration (20%) of the system studied preclude any growth in the system. Therefore, even in the event of brine chiller

contamination, rather than the brine chiller system becoming a continuous source of contamination, only that specific batch of brine could potentially contaminate the product.

It appears that varied pH and chlorine levels have little effect on the survival of *L. monocytogenes* 20% salt brine. Although this indicates that a brine chiller does not qualify as a hurdle that can reduce *L. monocytogenes* levels during the production of processed meats, this process realistically poses little risk as a source of post-cooking contamination.

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	pH	[Cl-]	Max. time	~ CFU level	Substrate tested
Test tube system	5-7	2,3	5 min	10^8	brine
	5	5,10,20	15 min	10^8	brine
	5,7	0,3	5 min	10^2	brine
Brine only in circulator	5,7	0,10	240 min	inoculum of 10^6 CFU	brine
Brine & hot dogs in circulator	5,7	0,10	30 min	inoculum of 10^3/ hot dog	brine
	5,7	0,10	30 min	inoculum of 10^3/ hot dog	hot dog

Table 1. Summary of the pH, Chlorine levels, time and inoculating dose of the systems tested

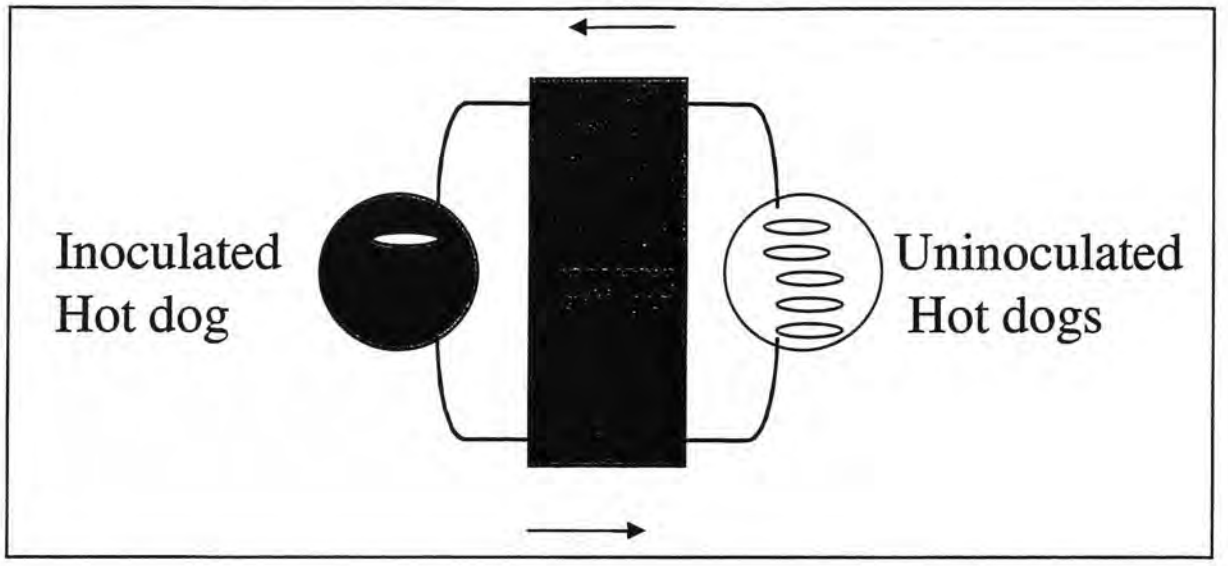

Figure 1. Schematic representation of the simulated recirculating brine chiller system

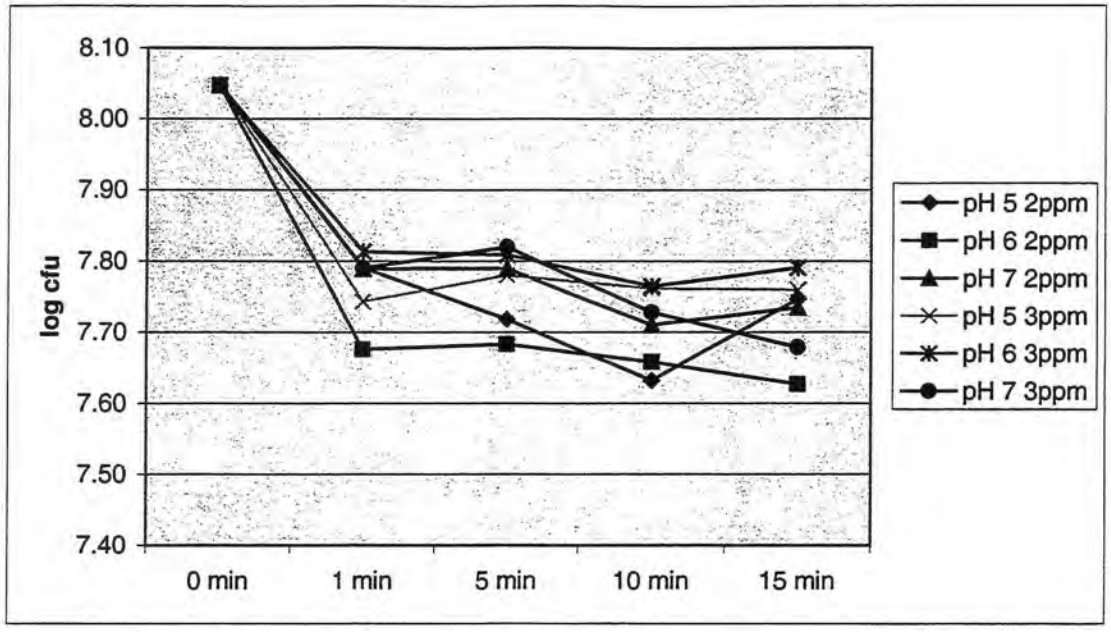

Figure 2. Graph of the average levels of *Listeria monocytogenes* in a test tube system with variable pH and 2 and 3 ppm free chlorine

General conclusions

Listeria monocytogenes represents an important food safety risk in the production of processed meats. The purpose of this research was to determine the effect of a recirculating brine chiller system on L. monocytogenes and to relate this information back to the danger of post cooking contamination.

In the test tube system with pHs of 5, 6, 7 and free chlorine levels of 2 and 3 ppm, with a high inoculation level (10^8), the organism exhibited an initial decline in numbers averaging 0.28 logs with no significance between any of the treatments. The slope of the following steady survival phase, while exhibiting a statistical difference for pH 7 with 3 ppm chlorine, was essentially equal to zero. Free chlorine levels of 5 and 10 ppm at pH 5 had a slightly higher reduction of 0.8 logs, but this reduction was still not biologically relevant. Significant reduction of L. monocytogenes was achieved using 20 ppm free chlorine, but this amount of chlorine is not feasible for use in industry. Evaluation of a level representing a realistic environmental contamination (10^2) exhibited a similar pattern with a 0.1 log initial reduction.

The recirculating system, at pH 5 and 10 ppm free chlorine, maintained a steady population of Listeria for an extended period of time. However, only low numbers of L. monocytogenes were recovered from the brine and uninoculated hot dogs.

The process of brine chilling processed meats does not reduce levels of Listeria monocytogenes and therefore cannot be considered a hurdle in improving the safety of processed meat production. However, it is important to realize that this does not necessarily make the process unsafe. In fact, the dilution effect observed during this study indicates that environmental contamination poses very little danger to the process. Therefore although brine chilling does not improve the safety of the process, it does not have the potential to be an important source of post processing contamination either.