Determining the Effect of Sodium Reduction on the Survival of *Listeria monocytogenes* and *Bacillus anthracis* in Cheddar Cheese

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Dedication

To my Mama and my Papa. I love you both very much!

Abstract

Sodium reduction from foods has been established as a public health priority. Cheddar cheese is a food targeted for this reduction because of its relatively high salt content of 600 mg sodium/100 g cheese. Among the roles of salt in Cheddar cheese, inhibition of spoilage and pathogenic microorganisms is a critical one. *Listeria monocytogenes* is a foodborne pathogen commonly found in dairy processing facilities that could easily contaminate Cheddar cheese. Additionally, a bioterrorism agent that is able to survive pasteurization, such as *Bacillus anthracis* could be intentionally added to cheese. The objective of this study was to determine the impact of sodium reduction on the ability of *L. monocytogenes* and *B. anthracis* to survive in Cheddar cheese.

Stirred curd Cheddar cheese was manufactured at full, reduced, and low sodium concentrations using two separate, single-strain starter cultures. In the reduced and low sodium treatments, curds received an application of either sodium chloride only, or sodium chloride with potassium chloride (KCl) to replace the effect of sodium chloride on water activity. After manufacture, cheese was analyzed for composition and aged. One week after manufacture, cheese samples were separately inoculated with *L. monocytogenes* and with spores of *B. anthracis* (at a level of 4 and 3 log CFU/g respectively), and stored at 4° or 12°C for 27 to 63 days. Microbiological testing of the cheese for pathogen survival, and natural microbial counts took place in three phases. Naturally present microorganisms included total aerobic count, lactic acid bacteria (LAB) count, and natural spore forming bacteria count.

The first stage of testing was run immediately after manufacturing, and simulates results of contamination during the cheesemake. During the first phase of testing, the *L. monocytogenes* population declined by 4 log CFU/g over 60 days of storage at 4°C. No difference based on starter culture or salt treatment was observed. The count of *B. anthracis* spores declined by 1.25 log CFU/g during this same storage period.

The second and third phases were conducted shortly after the completion of phase one and simulate post aging contamination of the cheese—both accidental and intentional. In the second and third phases of testing, the pH of the cheese had risen from an initial measurement of 4.7 to 5.1 and continued to rise throughout testing to 5.3. The survival of *L. monocytogenes* and *B. anthracis* was improved during these stages. The population of *L. monocytogenes* declined by only 1.5 to 2.5 log CFU/g (depending on stage), with few differences in population based on salt treatment. Under these same conditions, the *B. anthracis* spore counts declined by only 0.5 to 1 log CFU/g (depending on the stage), with few differences based on salt treatment. The survival of total aerobic, LAB and spore-forming bacterial counts was not affected by sodium reduction.

These data suggest that the low salt levels used in this study did not affect the survival of *L. monocytogenes* or *B. anthracis* at the experimental incubation temperatures, compared to controls. It follows that sodium reduction may not compromise the safety of food products like Cheddar cheese. These results may offer some assurance for the food industry to move forward in meeting sodium reduction initiatives. However, this progression must take place with caution. Although sodium reduction did not enhance the survival of the organisms, *L. monocytogenes* and *B.*

anthracis were still capable of surviving in Cheddar cheese. This observation stresses the requirement of pH control during cheese manufacture, adherence to good manufacturing practices, careful handling of products to prevent them from becoming contaminated in post processing operations such as slicing or shredding, and protection of product from intentional adulteration.

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Chapter 1: Introduction

Reduction of sodium intake is a public health priority. It is estimated that more than 75% of women and 95% of men in the United States exceed the daily upper intake level recommended for sodium, set between 1500 and 2300 mg per day, depending on demographics (Cohen et al., 2008). It has been well established that excessive consumption of sodium may lead to hypertension, cardiovascular disease, kidney failure, and in some cases bone cancer.

Because it has a relatively high salt content, Cheddar cheese has been targeted in sodium reduction projects. Efforts to control the level of sodium in Cheddar and other cheeses have been in place for several decades (Lindsay et al., 1982; Fitzgerald and Buckley, 1985; Ryser and Marth, 1987a; Schroeder et al., 1988; Mehta, 1994; Aly, 1995; Ayyash and Shah, 2011). Sodium chloride (salt) is an important ingredient used in the manufacture of Cheddar and other cheeses, as it is an important factor in both the overall flavor, and microbiological safety of the cheese. Furthermore, it is important to remember that sodium is an essential nutrient, and was originally beneficial—through the preservation (for increased shelf life) and safe-keeping effects it had on food; as such, strategies to remove sodium must maintain these original intentions (Doyle and Glass, 2010).

Many efforts have been put forth to develop low- or reduced sodium cheeses. The research on the effects of sodium reduction on the microbiological safety of the cheese has not been well documented (Taormina, 2010; Shrestha et al., 2011). Studies have shown survival of several pathogens in Cheddar cheese, including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* (Reitsma and Henning,

1996; Ryser and Marth, 1987a; Vera and L, 2003). An estimated 48 million Americans fall ill to foodborne illness each year (Scallan et al., 2011). Often this is a result of poor hygiene, or unsanitary manufacturing practices. Cheddar cheese could easily become unintentionally contaminated with *L. monocytogenes* because it is often found in dairy processing facilities.

Beyond the traditional concerns of unintentional contamination via pathogens in the environment (such as *Listeria monocytogenes*) lie concerns of intentional contamination of the United States (US) food supply. *Bacillus anthracis*, the etiologic agent of the disease anthrax is not typically considered a food pathogen. However, in 2001 when several citizens in the US received envelopes containing viable anthrax spores, the concern of intentional attack, especially on the US food supply, was heightened.

The primary goal of this research was to determine the effect of sodium reduction on the microbiological safety of Cheddar cheese. To accomplish this goal, stirred curd Cheddar cheese was manufactured with two sodium reduction levels—with and without potassium chloride added to compensate for sodium removal. Sodium reduction levels of 25 percent and 55 percent were chosen in accordance with the respective "reduced sodium" and "low sodium" claims that could be made (FDA, 2009; Johnson et al., 2009). After manufacture, the cheese was inoculated in order to determine the ability of *Listeria monocytogenes* and *Bacillus anthracis* to survive and the potential to grow or increase in number in Cheddar cheese, reduced sodium Cheddar cheese, and low sodium Cheddar

cheese (with and without the salt replacer potassium chloride) during storage at 4°C and 12°C.

It was also important to monitor the composition of the cheese and the natural background flora present during storage. These requirements were met by monitoring the presence of aerobic organisms, lactic acid bacteria and natural spore forming organisms over time in the aforementioned cheeses, when cheeses were stored at 4°C and 12°C as well as determining the chemical properties of each cheese.

Two hypotheses were developed:

- H_0: Sodium reduction and with and without substitution by potassium chloride will have no effect on the survival of *Listeria monocytogenes* or *Bacillus anthracis* in Cheddar cheese
- H_a: Sodium reduction with and without replacement by potassium chloride will have an effect on the survival of *Listeria monocytogenes* or *Bacillus anthracis* in Cheddar cheese. Increased sodium reduction will support increased survival

Chapter 2: A review of *Listeria monocytogenes*, *Bacillus anthracis,* food defense Cheddar cheese, and sodium reduction

2.1: *Listeria monocytogenes*

Listeria monocytogenes is a ubiquitous pathogen. It may be found in plants and soil, on farms, in infected animals or untreated manure; and in manufacturing facilities, in both humans and animals (Farber and Peterkin, 1991; Lund et al., 2000; Montville and Matthews, 2008). It is of particular interest as a foodborne pathogen, because of its ability to survive across a wide range of temperatures, including refrigeration temperatures (0 - 50°C) (Farber and Peterkin, 1991). *L. monocytogenes* has been cited as the foodborne pathogen with the third highest mortality rate in the United States (Scallan et al., 2011). *Listeria monocytogenes* has 13 serovars, but 95% of strains isolated from victims of listeriosis belong to serovars 1/2a, 1/2b or 4b (Farber and Peterkin, 1991; Montville and Matthews, 2008).

2.1.1: Bacterial characteristics

There are six species of the genus *Listeria: L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi*. *L. monocytogenes* is a Gram positive, non-spore forming facultative anaerobic rod. This species is oxidase negative and catalase positive, and produces clear zones on blood agar through the expression of β-hemolysis. *Listeria monocytogenes* is well known for its tumbling motility, a result of its flagella. This motility occurs primarily at temperatures ranging from 20° - 25°C, and is nearly undetectable at 37°C (Lund et al., 2000).

L. monocytogenes may grow in an environment with a pH as low as 4.4 (and may survive at levels below 4.3), and salt concentrations up to 12%. As a result of its ability to grow and survive in a wide variety of environments, *L. monocytogenes* is an organism of particular concern in products with a long shelf life, and products stored at refrigerated temperatures, such as cheese, fermented meat products, and ready-to-eat meats and cheeses.

2.1.2: Virulence factors

L. monocytogenes has multiple virulence factors that are regulated by its positive transcription factor (Kuhn et al., 1988; Farber and Peterkin, 1991; Liu, 2008). The first stage of attacking a host cell is completed by internalin and a protein designated p60, found in all virulent strains of *L. monocytogenes* (Hess et al., 1995; Labbe and Garcia, 2001; Liu, 2008). Once it has entered a host cell, *L. monocytogenes* secretes listeriolysin O: it works to stimulate intracellular growth, and detection of T-cells (Farber and Peterkin, 1991). *L. monocytogenes* is transmitted from cell to cell, rather than traveling outside of a cell to enter another cell. That is, once it enters a host cell, the organism will grow and divide within the cell before passing directly into neighboring cells. Moreover, it is able to utilize host cell actin proteins to enter vital organs, such as the brain or the placenta, through cell-to-cell transmission (Lund et al., 2000; Montville and Matthews, 2008). The use of the host cell actin proteins is accomplished after the secretion of actin polymerase (Shetron-Rama et al., 2002). Furthermore, the spread of *L. monocytogenes* among cells is aided by phospholipase C and phosphatidylinositol phospholipase C (Liu, 2008). Because of this unique transmission mechanism, the chance of *L. monocytogenes*

being exposed to host defenses is reduced, and thus the ability of the organism to survive is increased.

It has been reported that the virulence of *L. monocytogenes* may increase when it is grown at temperatures of approximately 4°C, thereby increasing the vulnerability of foods stored at refrigerated temperatures (Schlech III et al., 1983; Chakraborty and Goebel, 1988). Low storage temperatures often inhibit the survival of competing organisms, allowing for the proliferation of *L. monocytogenes,* especially in a product with a long shelf life (Linnan et al., 1988; Montville and Matthews, 2008).

2.1.3: Diseases caused by *Listeria monocytogenes*

2.1.3.1: Listeriosis

Listeria monocytogenes is the causative agent of listeriosis—a disease that infects both humans and animals. While all 13 serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 5, 6a, 6b) are able to induce listeriosis, 95% of foodborne cases are caused by serovars 1/2 a, 1/2 b, and 4b (Farber and Peterkin, 1991; Labbe and Garcia, 2001; Montville and Matthews, 2008). Animals carrying *L. monocytogenes* may infect humans or other animals when the bacteria are shed into milk, blood or feces (Farber and Peterkin, 1991; Marshall, 1992; Donnelly, 2004). Asymptomatic animal carriers of *L. monocytogenes* may shed the bacteria in fecal matter, which in turn may contaminate crops that are fertilized with manure (Nightengale et al., 2004).

L. monocytogenes is considered an extremely common environmental pathogen, and may also infect humans through exposure to or consumption of raw and processed dairy products, and ready-to-eat meat products (Marshall, 1992; Limjaroen et al., 2005). It is important to note that exposure does not imply that an infection will follow (Labbe

and Garcia, 2001). Post processing contamination is a common route for dissemination to processed products. This includes unsanitary manufacturing and cleaning practices at farm and manufacturing levels, as well as improper hygiene practices among employees. Figure 1 illustrates different routes of transmission of *L. monocytogenes* in a typical food supply chain (in this case, cheese manufacturing).

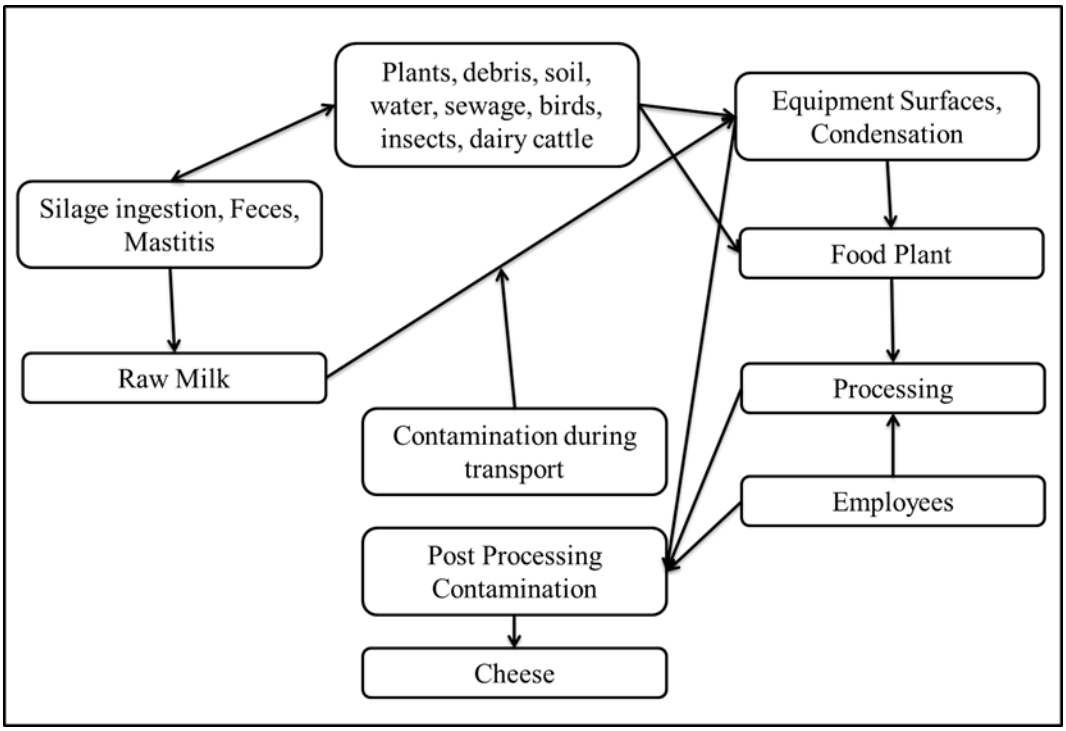

Figure 1: Possible routes of transmission of *Listeria monocytogenes* along the cheese supply chain. Adapted from (Lund et al., 2000).

Most cases of listeriosis in humans are sporadic and occur in individuals with a suppressed immune system; however, a generally healthy individual may develop listeriosis, or an individual can be an asymptomatic carrier (Farber and Peterkin, 1991; Lund et al., 2000; Labbe and Garcia, 2001; Montville and Matthews, 2008). Listeriosis may be divided into two categories: invasive, and noninvasive. The noninvasive

infection has an incubation time of 18 to 20 hours and results in typical gastroenteritis-like symptoms: fever, fatigue, cramps, nausea, vomiting (Labbe and Garcia, 2001). Upon development of invasive listeriosis, symptoms become much more severe and may include symptoms such as bacteremia, endocarditis, and various central nervous system infections, including meningitis and encephalitic infections (Farber and Peterkin, 1991; Labbe and Garcia, 2001). Most cases of listeriosis may be treated with antibiotics (Farber and Peterkin, 1991).

Identifying the food source of an invasive infection caused by *L. monocytogenes* is very difficult. The incubation time for the bacterium may be up to five weeks. With such a long time between ingestion and presentation of symptoms, food attribution may not be reliable, and the cause of listeriosis may go unknown. Furthermore, there is significant disagreement about the infectious dose of *L. monocytogenes*. Therefore, it is possible that a small dose of *L. monocytogenes* may contaminate a food product and go unnoticed during manufacturing, but grow to an infectious amount during distribution and storage. Thus, results from testing food products at the end of manufacturing are not certain indicators of the absence of *L. monocytogenes* from the food product.

2.1.3.2: Neonatal listeriosis

As previously noted, *L. monocytogenes* utilizes a unique method of dissemination among human cells. As a result, it is easily able to enter the placenta and potentially gain access to a fetus. If a woman is a carrier of *L. monocytogenes*, or becomes exposed to the bacteria while pregnant, a series of different consequences may occur. She may give birth to a healthy infant, or she may develop maternal listeriosis. In the event of the

latter, the mother may often suffer a series of influenza-like symptoms, and risks abortion, stillbirth, preterm labor and may also pass the listeriosis on to her newly born child (Farber and Peterkin, 1991; Labbe and Garcia 2001). In the event that a pregnant woman is infected with *L. monocytogenes*, the mother is most often infected in her final trimester (Lund et al., 2000). Neonatal listeriosis is classified as one of two subtypes: early-onset or late-onset. Early-onset results from an in utero infection, which leads to septicemia, and usually causes infant mortality at a rate of 15 to 50% (Farber and Peterkin, 1991). Early-onset most often occurs within 0 to 2 days of birth (Lund et al., 2000). Late-onset is characterized by meningitis, usually within 5 days of birth, and results in a mortality rate of 10 to 20% (Farber and Peterkin, 1991).

2.1.4: Epidemiological relevance

An estimated 1600 cases of listeriosis occur each year in the United States, resulting in 1,455 hospitalizations, 255 deaths, and costs of over 2 billion dollars in healthcare associated expenses (Scallan et al., 2011; Scharff, 2012). Although there has been a decline in the relative rate of *Listeria monocytogenes* related infections over the past ten years, it still ranks among the top three causes of foodborne related deaths in the United States each year (Maki, 2006; Scallan et al., 2011). Most recently, a multi-state outbreak of listeriosis occurred in 2011, and was ultimately linked to whole cantaloupes grown by Jensen Farms, in Colorado. There were 146 cases of listeriosis related to this outbreak, resulting in the deaths of 34 people.

The FDA outlined several factors that contributed to this outbreak. It is possible that there was a low level of *L. monocytogenes* present in the fields in which the

cantaloupes were grown. This low level of contamination could have then been introduced into the processing facilities. Examination of the farm and processing facilities indicated that the floors and packing areas were difficult to clean and allowed water to pool in several areas. Furthermore, there was no step in the processing of cantaloupes to lower their temperature after harvesting and before refrigerated storage. It is likely that this gradual temperature change promoted the collection of condensation, and the growth of *L. monocytogenes* (CDC, 2011). The cantaloupe outbreak was the first large-scale outbreak of listeriosis to be linked to fresh produce. Previously, only ready-to-eat meats and cheeses were associated with outbreaks of this magnitude. A list of additional foodborne outbreaks of *L. monocytogenes* may be seen in Table 1.

Table 1: A brief history of foodborne illness outbreaks associated with *L. monocytogenes*

Foodborne illness outbreaks associated with *L. monocytogenes*			
Year	**Food Source**	**Cases (Deaths)**	**Source**
1983	Milk (pasteurized)	49 (14)	(Fleming et al., 1985)
1985	Mexican-Style Cheese	142 (48)	(Linnan et al., 1988)
1986	Ice Cream	36 (16)	(Lund et al., 2000)
1998	Hot Dogs	79 (21)	(CDC, 1998)
2003	Queso Fresco	12 (1)	(CDC, 2012b)
2011	Cantaloupes	146 (34)	(CDC, 2011)

2.1.5: Incidence of *Listeria monocytogenes* in cheese

Cheese has been considered a product relatively susceptible to post-processing contamination with *L. monocytogenes* because the organism may be found in raw milk, because cheese is exposed to the plant processing environment after thermal processing, and because most varieties of cheese are ready-to-eat products (Farber and Peterkin,

1991; Lund et al., 2000; Trinetta et al., 2012). *L. monocytogenes* is able to survive and grow in cheese because it is able to thrive in refrigerated conditions, and in environments of moderate salt concentrations and mild pH—conditions that may be typical in Cheddar cheese (Montville and Matthews, 2008). If present during cheese manufacture, Farber and Peterkin (1991) reported that *L. monocytogenes* was found in higher amounts in the curd than in the whey.

One of the largest recorded and most frequently cited outbreaks of *Listeria monocytogenes* was caused by contaminated cheese in southern California in 1985. This epidemic was linked to Mexican-style cheese made with improperly pasteurized milk. Over the course of eight months, a 4b serotype of *L. monocytogenes* caused 142 cases of human listeriosis, resulting in 48 deaths (Linnan et al., 1988). While many manufacturing sites follow standard safety and sanitization procedures there are multiple possible points of entry for *L. monocytogenes* into the cheese supply chain; extra vigilance must be practiced in order to produce an end product safe for consumption. See figure 1 for an overview of possible paths of transmission of *L. monocytogenes* during the manufacture of cheese.

Since that outbreak in 1985, the CDC has augmented its record-keeping and reporting procedures both for foods contaminated with *L. monocytogenes* and for reported cases of listeriosis. The National Outbreak Reporting System (NORS) was launched by the CDC in 2009 to maintain data of reports of foodborne illness outbreaks. These data are reported in the Foodborne Outbreak Online Database (FOOD). A review

of FOOD shows seven outbreaks in the United States linked to *L. monocytogenes* in cheese from 1998 to 2009.

Among them is a multistate outbreak of listeriosis that occurred between October 2008 and March 2009. The outbreak resulted in eight cases of listeriosis, three hospitalizations, and two stillbirths, all occurring in Hispanic women (Jackson et al., 2011). The Michigan Department of Agriculture (MDA) determined that the outbreak was caused by asadero cheese that had been made with pasteurized milk. Upon further inspection, investigators determined that the milk was contaminated after pasteurization by a vat gasket that had been improperly cleaned. The manufacturing plant responsible for the incident was shut down until the product associated with the outbreak was destroyed, and the vat gasket was removed (Jackson et al., 2011).

Close review of the previously listed outbreaks indicates that the cheeses implicated were made with raw milk, or pasteurized milk that was contaminated after pasteurization, often due to improperly cleaned manufacturing parts. This pattern highlights the importance of proper manufacturing, cleaning, and sanitizing practices in the manufacture of cheese. Additionally, these outbreaks underline the importance of careful monitoring of cheese for contamination by *Listeria monocytogenes*.

2.2: *Bacillus anthracis*

2.2.1: Microbial characteristics

Bacillus anthracis is a Gram-positive, facultative anaerobic, spore-forming, rod shaped bacteria. As a spore-forming organism, *Bacillus anthracis* transforms itself through a series of cellular changes—activation, germination, and outgrowth—during its

development from dormant spore into vegetative cell. Germination may begin once adequate nutrients are provided. During outgrowth, Moberly et al. (1966) were some of the first that observed development of a new vegetative cell, and its division from the spore.

2.2.2: Physiological properties

It has been frequently noted that *B. anthracis* is a bacterial species that is closely related to *B. cereus* (Moberly et al., 1966; Mock and Fouet, 2001; Vilas-Boas et al., 2007). One of the defining characteristics that differentiate the two species is the ability of *B. anthracis* to form a capsule and produce toxins. A secondary differentiating characteristic between the two species is the difference in susceptibility to the antibiotic penicillin—*B. anthracis* is susceptible, while *B. cereus* is not (Lund et al., 2000). *B. cereus* and *B. anthracis* both contain the regulator PlcR, responsible for the regulation of nearly one hundred genes related to the virulence of the strains (Vilas-Boas et al., 2007). Vilas-Boas et al. (2007) reported that the genes regulated by PlcR were present in *B. anthracis*, but due to a nonsense mutation, the *plcR* gene corresponding to the regulon is not always expressed. This latter characteristic is another difference between *B. cereus* and *B. anthracis*.

2.2.3: Sporulation

The level of efficiency observed for sporulation is heavily influenced by environmental conditions, with the process of sporulation beginning in an environment of extreme nutrient deprivation. Following the life cycle of a typical spore former, the

terminal point in the life cycle of *Bacillus anthracis* is the spore formation. The new spore may remain dormant for many years, or germinate immediately depending on the environment (Moberly et al., 1966; Mock and Fouet, 2001). When dormant, the spores of the organism are extremely resistant to adverse environmental conditions, including high temperatures, high pressure, chemical agents, and varying forms of radiation (Mock and Fouet, 2001; Vilas-Boas et al., 2007).

2.2.4: Virulence factors

Two virulence factors of *Bacillus anthracis* are its tripartite toxin, and its capsule (Mock and Fouet, 2001; Fouet and Mock, 2006). The toxin causes toxemia and the capsule causes septicemia in infected persons (Fouet and Mock, 2006). *B. anthracis* has two plasmids responsible for virulence: the pXO1 and pXO2 plasmids. Because these two plasmids contain the primary virulence factors for *B. anthracis*, it is possible (and common) to produce non-virulent strains through the removal of one or both plasmids (Vilas-Boas et al., 2007). It is possible to cure the organism of its plasmids through the growth of strains at 43°C, or growth in the presence of antibiotics (Mock and Fouet, 2001). The gene *atxA* is seen as the master regulator within *B. anthracis*, and enhances the efficacy of the virulence factors listed above (Fouet and Mock, 2006).

2.2.4.1: pXO1 plasmid

The pXO1 plasmid carries the protective antigen (PA). The PA is able to release both the lethal factor (LF) and the edema factor (EF) (Fouet and Mock, 2006). The LF is a zinc protease, and the EF is an adenylate cyclase. The PA combines with either the LF or the EF to cause human death or edema in the skin respectively (Mock and Fouet,

2001). Interestingly, when introduced to the host separately from each other (i.e., *only* the edema factor or *only* the lethal factor), these proteins are not toxic (Mock and Fouet, 2001). Rather, once the PA and LF begin to associate with each other, they produce an exotoxin, named the lethal toxin (LTx), which is able to cause lethal shock (Vilas-Boas et al., 2007). Additionally, the PA and EF may come together to form an exotoxin named edema toxin (ETx), responsible for the production of edema in the skin (Vilas-Boas et al., 2007).

The protective antigen has four domains, each responsible for a different step in the process of intoxication within the host. The first domain holds the proteolytic activation site, the second is responsible for pore formation, the third aids in protein-protein interactions, and the fourth is required for the protective antigen to bind to the receptor. In addition to the PA, LF, and EF, the pXO1 plasmid carries the operon *gerX*, responsible for the germination of *B. anthracis* spores (Mock and Fouet, 2001).

2.2.4.2: pXO2 plasmid

The pxO2 plasmid is responsible for the synthesis and degradation of the *B. anthracis* capsule (Mock and Fouet, 2001). Since the major component of the capsule is poly-γ-D-glutamate, amino acids are the primary structural component of the capsule (Fouet and Mock, 2006). This configuration is rather unique; most other bacterial capsules are made up of a series of polysaccharides (Choudhury et al., 2006). It is the capsule that allows *B. anthracis* to outlast immune defense mechanisms used by the host; additionally, the capsule is one of the primary factors leading to septicemia (Mock and Fouet, 2001).

2.2.5: Diseases caused by *Bacillus anthracis*

2.2.5.1: Anthrax

B. anthracis is the etiological agent of anthrax, a disease occurring mainly in herbivores, but able to infect all mammals (Mock and Fouet, 2001; Fouet and Mock, 2006). A host will contract the disease after *B. anthracis* spores have entered into the host body and are transported to the lymph nodes by macrophages; eventually the lymph nodes become overwhelmed by the infection, and the bacilli will enter the bloodstream (Mock and Fouet, 2001).

2.2.5.1.1: Cutaneous anthrax

Cutaneous anthrax is the most common form of the disease and accounts for more than 90% of all human cases of anthrax (Vilas-Boas et al., 2007). It presents in the host in the form of a small pimple that eventually turns to a black eschar, and may cause edema (Mock and Fouet, 2001). Because of these easily distinguishable symptoms, this form of anthrax is quite simple to diagnose; as a result, it can be treated fairly quickly with antibiotics (Mock and Fouet, 2001; Vilas-Boas et al., 2007).

2.2.5.1.2: Inhalation anthrax

Inhalation anthrax—also known as pulmonary anthrax—traditionally involves two distinct stages. The first stage is characterized by mild symptoms, such as gastroenteritis, flu-like symptoms and fever (Plotkin et al., 1960; Mock and Fouet, 2001). As the disease enters the second stage, it becomes extremely aggressive and resistant to treatment, presenting symptoms such as sepsis and respiratory failure (Mock and Fouet,

2001). It is common for the first stage to last multiple days, and rare for the second to last more than 24 hours before death occurs. Because the first stage can so often be mistaken for influenza or similar infections, it is relatively difficult to diagnose before death (Plotkin et al., 1960).

2.2.5.1.3: Gastrointestinal anthrax

Gastrointestinal (GI) anthrax develops after the consumption of *Bacillus anthracis* spores. Meat from animals previously infected with anthrax is the most common cause of GI anthrax in humans (Leishman et al., 2010b). As in cases of pulmonary anthrax, GI anthrax may be difficult to diagnose early on, but becomes fatal very quickly (Mock and Fouet, 2001). Because the disease is characterized by symptoms such as fever, nausea, bloody diarrhea, and vomiting, it is easy to see how one may not immediately associate symptoms with anthrax but with influenza-type diseases instead (Vilas-Boas et al., 2007).

2.2.6: Epidemiological relevance

While cases of anthrax are quite rare, they are reported sporadically. In the United States, anthrax infections are most commonly contracted from infected animals or animal products that have been contaminated to human workers (Jernigan et al., 2002). In 1957 during a 10 week period, five cases of inhalation anthrax and 4 cases of cutaneous anthrax occurred (Plotkin et al., 1960). This epidemic broke out among employees of an industrial plant that handled large amounts of wool, in a wool sorting operation. While all four employees with cutaneous anthrax accomplished a full recovery through the use of antibiotic regimens, only one employee diagnosed with inhalation anthrax survived (Plotkin et al., 1960).

In 2000, anthrax was detected in a steer carcass in Roseau, Minnesota. The carcass belonged to a small family-owned farm, and was slaughtered after being deemed acceptable for slaughter by a veterinarian. After slaughter, the family used the carcass to make hamburgers and steaks, and consumed these items within three weeks of slaughter. Shortly after consumption, family members reported symptoms of gastrointestinal illness and one member reported a fever of 102°F. At that time, samples of the carcass were tested for and were confirmed as containing *B. anthracis* and the family was immediately given treatment with antibiotic prophylaxis with ciprofloxacin (CDC, 2000). This episode did not result in any deaths, but the timing of confirmation of *B. anthracis* in the carcass was crucial to the safety of the family and surrounding community members who may have used the same slaughter facilities.

Perhaps the most recent case of anthrax in the United States occurred in the fall of 2001. This outbreak spanned from October 4 to November 20, 2001 and included 11 cases of cutaneous anthrax, 11 cases of inhalation anthrax, and unfortunately led to five deaths. This outbreak was extremely unique, as it was the first bioterrorism-related case of anthrax in the United States (Jernigan et al., 2002). While anthrax is normally contracted through exposure to infected animals and sometimes humans, in this outbreak, most of the cases were contracted when patients received an envelope in the mail containing a powder with *Bacillus anthracis* spores, or were exposed to these envelopes through their work in the U.S. Postal Service.

With increasing threats of bioterrorism, it is interesting to note that a vaccine for anthrax is not readily available to the general public. Immediately after the anthrax mail

scare in 2001, instead of vaccination, the Centers for Disease Control and Prevention (CDC) recommended an antimicrobial treatment to prevent inhalational anthrax. The CDC concluded that the immediate use of this procedure was effective and can prevent disease (Jernigan et al., 2002). Most vaccines for anthrax are developed through the use of *B. anthracis* strains from which the pXO2 plasmid has been removed (Vilas-Boas et al., 2007). Currently, a vaccine is available, but is recommended for very particular circumstances, such as a career in which one may be exposed to anthrax often, or a military assignment in which exposure to anthrax is of high concern (CDC, 2010). The vaccine does not contain live bacteria; rather, the primary ingredient of the vaccine is the protective antigen (DOD, 2010). In efforts to prevent the spread of *B. anthracis* and development of anthrax, rather than reliance upon a vaccine, a safer option may be to screen at-risk foods and products with a hand-held polymerase chain reaction (PCR) unit (a unit for rapid detection) developed for the organism (Montville and Matthews, 2008).

B. anthracis is considered an ideal biological weapon, and was of high concern during the Cold War. The United States, the former Soviet Union, and other countries developed stockpiles of weaponized spores. In 1972, the United Nations opened for signature The Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction; commonly known as the Biological Weapons Convention (BWC). After being signed by 103 countries, the United Nations entered this convention into use in 1975. This mandated the destruction of all stockpiles (United Nations, 1975).

B. anthracis is classified as a category A bioterrorism agent by the CDC, and could potentially be used as a weapon through the contamination of the United States food supply. A response to this type of attack would be wide-ranging in its approach, and likely come in multiple stages (Hilgren et al., 2007). While an aerosolized attack with dried *B. anthracis* spores is more likely, an intentional contamination of the food supply is certainly possible, and would absolutely lead to devastating consequences (Wein and Liu, 2005; Leishman et al., 2010b).

2.3: Food defense

2.3.1: A new vocabulary

At one time, terms such as "food safety," "food defense," "food protection," and "food security" were used somewhat interchangeably (Ackleson et al., 2011). In a discussion of vulnerabilities of a particular food system or an intentional attack on a food system, it is important to differentiate between the terms. "Food safety" generally refers to specific actions that may be taken, or policies that may be put into place to prevent natural or accidental contamination of food and food products. "Food defense" is a term regarding practices implemented to avoid an episode of intentional contamination of a food supply or a food manufacturing facility. "Food protection" is used most often as an over-arching term that accounts for both "food safety" and "food defense" measures. Perhaps the most interesting of these expressions is "food security." Traditionally, in developing countries, this term refers to the practice of producing and preserving an adequate amount of food so that all members of a society will be able to eat (Busta and Kennedy, 2011). In developed nations, "food security" is seen as another all-

encompassing term to describe efforts that can be divided into "food safety" and "food defense" (Ackleson et al., 2011). An increase in recent discussions of the definitions of these terms points to an increased awareness of the threat of intentional attacks on the U.S. food supply.

2.3.2: Intentional attacks on the U.S. food supply: a brief history

As defined by the World Health Organization (WHO), food terrorism is "an act or threat of deliberate contamination of food for human consumption with biological, chemical and physical agents or radio-nuclear materials for the purpose of causing injury or death to civilian populations and/or disruption of social, economic or political stability" (Department of Food Safety, 2008). After the terrorist attacks on September 11, 2001, concerns regarding the vulnerability of the food supply in the United States increased. In the years following the attacks, several Department of Homeland Security Presidential Directives (HSPDs) were issued in an effort to outline and establish various practices that would increase the safety of the citizens of the United States. One of particular interest was issued in 2004, HSPD 9: Defense of the United States Agriculture and Food. HSPD 9 is a series of national policies put in place to protect the U.S. food supply (DHS, 2004). These policies consider natural disasters, intentional terrorist-based attacks, and accidental introduction of pests or insects, among other threats. The introduction of this directive shows that the farm-to-fork continuum is indeed vulnerable, but that those in the highest offices of our nation have ordered that the food supply be fiercely protected from an intentional attack.

Traditionally, the concept of an intentional attack on the U.S. food supply has been considered relatively foreign to American citizens. Even so, there have been documented cases in recent history. During the last 15 years, there have been cases of intentional attack on the U.S. food supply displaying the use of 11 different substances (Busta and Kennedy, 2011). Consider the first, an outbreak of *Salmonella* Typhimurium as a result of the intentional contamination of the salad bar at Shakey's Pizza and several other restaurants in The Dalles, Oregon. Between 1981 and 1984, a religious cult known as the Rajneeshees, led by Bhagwan Shree Rajneesh worked to build a small town named Rajneeshpuram near The Dalles (MacDonald, 2011). Over this time period, the cult members established their own police force, and even began to take over neighboring towns by winning electoral control (Ackleson et al., 2011).

In September 1984, several members of the local community began displaying symptoms such as stomach cramps, vomiting, and diarrhea (Miller et al., 2002). Over the next few months, more than 1,000 cases of similar symptoms were reported to local health officials, and 751 were confirmed as cases of salmonellosis caused *Salmonella* Typhimurium (MacDonald, 2011). Due in part to fear of being accused of persecution, local public health officials resisted accusing the Rajneeshees of introducing the contamination, even after multiple local restaurants showed positive results for the same strain *Salmonella* Typhimurium, one that was not commonly present in previous outbreaks (Ackleson et al., 2011). Residents of the small town in Oregon had to wait nearly a year before internal struggles of the cult began to surface, culminating in Bhagwan Rajneesh accusing members of his cult of attempting to poison members of the

local community. After calling a press conference and making his accusation in public, an investigation was called. Eventually, the Federal Bureau of Investigation found the cult guilty of maintaining cultures of *Salmonella* Typhimurium in their lab in Rajneeshpuram, as well stocks of poisons capable of slowly killing humans and animals (Miller et al., 2002). Upon the completion of the investigation, Rajneesh, and two other leaders of the Rajneeshees implicated in the case, served sentences and then fled the country (Ackleson et al., 2011; MacDonald, 2011).

The act of intentionally contaminating food may be one of malicious intent, but may also be executed impulsively. In 2003, a disgruntled grocery store employee caused an episode of intentional contamination. He was indicted for the contamination of over 200 pounds of ground beef that was sold to consumers from a grocery store in Michigan (CDC, 2003). Consumers who purchased the ground beef complained of symptoms almost immediately after consuming the product. These symptoms included dizziness, vomiting, nausea, and burning of the mouth. After a regional medical center returned presumptive results of nicotine contamination in the product, public health officials developed a case definition. After 148 interviews, officials found 92 cases of nicotine poisoning included in the outbreak (CDC, 2003). Ultimately, officials discovered that the employee of the grocery store had added Black Leaf 40—an insecticide made up of 40% nicotine—to the beef that was ground and sold in the grocery store (CDC, 2003).

These two examples of intentional attacks on the U.S. food supply serve as strong reminders of the importance of developing, implementing and maintaining strict food safety standards throughout the farm-to-fork continuum.

2.3.3: Governing agencies

The majority of the responsibilities for maintaining the safety of the U.S. food supply are distributed among the Food and Drug Administration (FDA), United States Department of Agriculture (USDA), and the Centers for Disease Control and Prevention (CDC). Rightfully so, as intentional contamination incidents are often large issues for the food industry. These organizations lend support to local regulatory bodies, such as state and regional public health departments that experience the consequences of foodborne illness outbreaks—both intentional and unintentional (Spink and Moyer, 2011).

In a society where an increasing proportion of information is accessed electronically, it is interesting to consider the possibility of these agencies using social media to augment foodborne disease surveillance systems. A recent study suggests the possibility of monitoring outbreaks virtually as they are happening, rather than taking a reactive approach based exclusively on sample collection, reporting, and other traditional methods (Newkirk et al., 2012). The study showed that the immediate access to social networking platforms allows consumers to give an accurate description of their personal state of health in practically "real time"; additionally, the study suggested that consumers may be more likely to publish updates to these outlets than to contact regulatory bodies such as local health departments.

The Department of Homeland Security (DHS) also holds some responsibility for the safety of the domestic food supply. With the implementation of the Public Health Security and the Bioterrorism Preparedness Act of 2002 (also known as the biosecurity act of 2002), the DHS became responsible for monitoring the imports and exports of food

to and from the United States. Additionally, the DHS gained responsibility for the prevention of bioterror acts, including those targeting food (FDA, 2002).

2.3.4: Vulnerabilities in the U.S. food supply and Cheddar cheese manufacture

Many would argue that the food system in the United States is among, if not the safest, in the world. The American food supply is governed by multiple agencies. These agencies use findings from scientific research and risk analysis to regulate the food system, despite constant pressure from citizens and activist groups to regulate based on public perception (Lofstedt, 2011). Even so, there is a very genuine threat of intentional contamination of the food supply in the United States that should not be ignored (Busta and Kennedy, 2011). In the last 50 years, advances in technology across the globe have allowed consumers access to a fairly consistent food supply regardless of geographic location or season. This luxury is the result of an increasingly complex and globalized food supply. With more and more food products, packages and ingredients coming from different areas of the world, increased points of access to the food supply are created. It follows then, that the ease of contaminating the food supply is enhanced through increased globalization. In response to this growing threat, it is absolutely critical that those organizations responsible for domestic and global food supplies work together to provide a continuously safe food system (Ackleson et al., 2011; Busta and Kennedy, 2011).

Ackleson et al. (2011) have stated the four most prominent factors that put the U.S. food supply at risk for an intentional attack: batch sizes that result in a number of servings large enough to attain morbidity or mortality; short retail shelf life or turnaround

rate that lead to rapid consumption and reduce the amount of time in which public health officials may respond; uniform mixing steps in food manufacturing that allow contaminants to be distributed evenly throughout a large batch; and ease of access granted to a relatively large number of individuals working in some part of the food supply chain (Busta and Kennedy, 2011).

2.3.4.1: Milk

The milk supply in the United States follows a bow-tie shaped line of supply (Wein and Liu, 2005). The milk from many cows is combined in holding tanks on a farm, further mixed with other farm holding tanks in a smaller number of milk trucks, brought to a smaller number still of processing lines to create one finished product—fluid milk. Because milk is a liquid product, it is relatively simple for a small dose of contamination added at one farm to spread quickly throughout the entire volume collected from a multitude of farms. The shelf life of fluid milk is quite short, and after collection and processing it is quickly redistributed to a large population that includes many at-risk individuals. An episode of intentional contamination of the milk supply would have devastating effects. While pasteurization is an important safety hurdle included in milk processing, it has been concluded that standard pasteurization practices are not severe enough to kill bioterror agents such as *B. anthracis* spores (Perdue et al., 2003; Xu et al., 2006). Moreover, the cost of testing for *B. anthracis* and other select agents that are not included in traditional surveillance systems is prohibitive—in some cases, in excess of $200,000,000 annually (Kennedy, 2008). In the event of an intentional contamination, even if spores were added to milk that was still to be pasteurized, because

milk is not routinely tested for the presence of *B. anthracis*, it is possible that the spores would be able to survive long enough to end up in a finished product—such as Cheddar cheese—and reach the consumer.

2.3.4.2: Cheddar cheese

When considering the safety of Cheddar cheese with respect to its microbial load, and pathogenic bacteria, it is important to review such factors as the quality of milk, native flora of the milk, type of starter culture used, pH during cheese manufacture and overall salt content (Donnelly, 2004). Pasteurized milk is used most often in the manufacture of Cheddar cheese. Because it is pasteurized, the milk itself does not pose a serious threat to the microbial safety of the cheese outside of an episode intentional contamination. However, one of the very first steps in the manufacture of cheese is to allow the fluid milk to come to an elevated temperature (usually 31°C), and further to subject the milk to a mixing step in an effort to fully incorporate the starter culture, color, and any flavor adjuncts.

As previously discussed, the physical nature of milk, combined with the holding and mixing steps of cheese processing provide an environment in which a small dose of contamination could quickly spread homogeneously through an entire vat, thereby contaminating the full batch of cheese. Although the inherent characteristics of Cheddar cheese usually result in a decline in the population of organisms like *L. monocytogenes*, it is important to keep in mind the possibility of cheese becoming contaminated through post-manufacturing contamination, especially by *L. monocytogenes* (Donnelly, 2004).

It is common to find *L. monocytogenes* in dairy processing plants; it can be found near coolers, in drain wells, on the floors and on the walls (Donnelly, 2004). Consider a batch of Cheddar cheese that is manufactured under clean conditions with no contamination. If it is aged before additional processing such as slicing or shredding followed by repackaging, these operations present added opportunities in which the cheese may be contaminated. For food defense purposes, organisms that may be used in intentional acts of contamination, such as *B. anthracis* could be added during the aforementioned mixing step(s) and survive both the cooking steps and long storage periods at refrigerated temperatures.

2.4: Cheddar cheese

2.4.1: History of Cheddar cheese

Fluid milk has a short shelf life; using it to manufacture cheese (and other products) is a way to use dehydration, acid and salt to increase its shelf life. The manufacture of cheese is used to give longer life to a commodity which would otherwise need to be disposed of after a short amount of time. It is likely that the very first cheese was produced accidentally. As the techniques for cheese production evolved, various types of cheeses were developed all over the world. One of the most popular, originated near Cheddar, England (Fox et al., 2000).

Cheddar cheese is the most commonly produced and second most commonly eaten cheese in the United States. It is now consumed at a rate of 10.44 pounds per capita and a total of 2.4 billion pounds of Cheddar cheese are produced each year in the United

States. Cheddar cheese production accounts for more than 75% of the natural cheese produced in the US (IDFA, 2011).

2.4.2: Composition and standard of identity

The standard of identity for Cheddar cheese is described in title 21, part 133 of the Code of Federal Regulations, and states that Cheddar cheese may be no more than 39% moisture, and must contain 50% milkfat in solids. The Code of Federal Regulations further states that the cheese must be made from cow's milk, whether from skim, cream or a combination thereof. Finally, the regulations state the ingredients like color, calcium chloride, salt, and enzymes may be used as optional ingredients (Code of Federal Regulations, 2006).

2.4.3: Processing and manufacture

While the use of pasteurization is certainly commonplace in the food industry today, the practice of heat-treating foods, and especially milk was not as common in the United States until 1924, upon the implementation of the Pasteurized Milk Ordinance (Marshall, 1992).

After pasteurization, the milk is brought to temperature (usually around 31°C), and the chosen starter culture is added. This temperature is used because it is near the optimum temperature for the starter culture and allows for activity by the coagulating enzyme. Starter cultures used in Cheddar cheese traditionally consist of members of the genus *Lactococcus*, such as *Lactococcus lactis* ssp. *lactis*, or *Lactococcus lactis* ssp. *cremoris.* The starter cultures may be in the traditional bulk culture form, or a more concentrated form, known as direct-vat-set (DVS). In either form, starter cultures are

chosen based on their resistance to phages speed of acid production, and heat stability, among other factors.

For example, starter cultures may be chosen based on the flavor they impart in the final product, with the hope of avoiding a bitter cheese (Emmons et al., 1962; Law, 1999). Starter cultures may also be selected for their tolerance to ingredients used in cheese making, such as salt. Turner and Thomas (1980) reported *L. lactis* ssp. *lactis* to be less salt tolerant than *L. lactis* ssp. *cremoris*. Organisms in the starter culture convert lactose in the milk into lactic acid, creating an ideal environment in which the coagulating enzyme(s) may function. As the cheese ages, these organisms will be unable to survive due to high salt concentrations, low pH, and their consumption of the majority of lactose present (McSweeney et al., 1993).

After adding the starter culture, the next step in manufacture is to coagulate the casein proteins. In modern manufacture of Cheddar cheese, this is most commonly performed enzymatically, using recombinant chymosin. Before recombinant chymosin was readily available for cheese making, rennet was used to coagulate the milk during cheese making. Rennet is product obtained from the abomasum of young calves that contains a mix of digestive enzymes (Fox et al., 2004). When first added to the milk, the chymosin enzyme (whether recombinant or from rennet) attacks kappa-casein (Walstra, 1999). The casein micelles are virtually instantaneously attracted to calcium ions. As bonds form between the casein micelles and the calcium ions, a network forms to create a gel in which the milk fat is trapped in the casein matrix (Linklater, 1966; Law, 1999). While the gel is being formed, lactic acid bacteria continue to produce lactic acid. As

acidity increases, so does the activity of the rennet, thereby increasing the rate of gel formation (Linklater, 1966).

Once a coagulated gel is fully formed, the mass is cut into curds. When cut, the casein network tightens and the curds begin expelling whey—a phenomenon known as syneresis—and start to 'heal', or form a skin made up of a layer of compressed casein micelles (Law, 1999). The expelled whey contains the components of the milk that are water soluble: some lactose, whey proteins and minerals, and may contain small amounts of fat that are lost during the initial expulsion and before the formation of a skin (Law, 1999).

Once the curd has been cut, it is cooked: a process in which the curd is stirred, usually for 60 to 80 minutes while increasing the temperature from 88°F to 102°F (time and temperature combinations are fairly subjective and often determined based on the look and feel of the curds as observed by the cheese maker). This step has a profound effect on the net syneresis. During this cooking step, the starter culture will continue to produce lactic acid, causing the pH to continue to drop. A greater drop in pH, higher temperature used during cooking or high rate of stirring may contribute to increased expulsion of whey (Law, 1999).

In the manufacture of stirred curd Cheddar cheese, after the curd has been cut and cooked, the whey is drained. Initially, half of the whey is drained off, while the remaining volume is allowed to rest with the curd. Reducing the volume of whey results in an increase in the number of collisions between curds as they are being agitated;

increased agitation results in increased whey expulsion and the firming of the curd more quickly (Linklater, 1966).

Perhaps the most crucial processing step in the manufacture of Cheddar cheese is what makes it different from other, similar types of cheese: cheddaring. This step is only performed in the manufacture of Cheddar cheese, but in the manufacture of stirred curd Cheddar cheese, it is omitted. During cheddaring, as whey drains, the curds begin to mat together. Once the whey has been removed, curds are allowed to mat together and cut into blocks, and blocks are stacked on top of each other on the sides of the cheese vat, leaving the center of the vat open for further expelled whey to drain. The blocks are then flipped, rotated, and re-stacked several times (in approximately ten to 15 minute intervals) over the course of approximately two hours (Linklater, 1966). The purpose of Cheddaring is to develop the texture and the acidity of the final product.

After the Cheddaring is completed, the blocks of curd are milled into small pieces. Once the curd has been milled, it may be salted, pressed into hoops that have been dressed with cheesecloth, and aged as desired. While the amount of salt added to each batch of cheese is based in part on previous experience of the cheese-maker, most Cheddar cheese contains approximately 1.7 to 2.1% salt (Johnson et al., 2009).

The salting process used during the manufacture of stirred curd Cheddar cheese is somewhat different. Instead of allowing the curds to mat together, after the whey is drained, the curds are salted and directly placed into hoops for pressing. The different salting procedures result in different amounts of salt diffusion into the final products. Because the salt application in the manufacture of Cheddar cheese takes place after

milling, the surface area exposed to the salt is less than that of stirred curd Cheddar curds. Therefore, the amount of time required for the salt to reach the center of a Cheddar cheese curd is greater than that required for a curd of stirred curd Cheddar cheese. For a figure summarizing the production of Cheddar cheese, see figure 2.

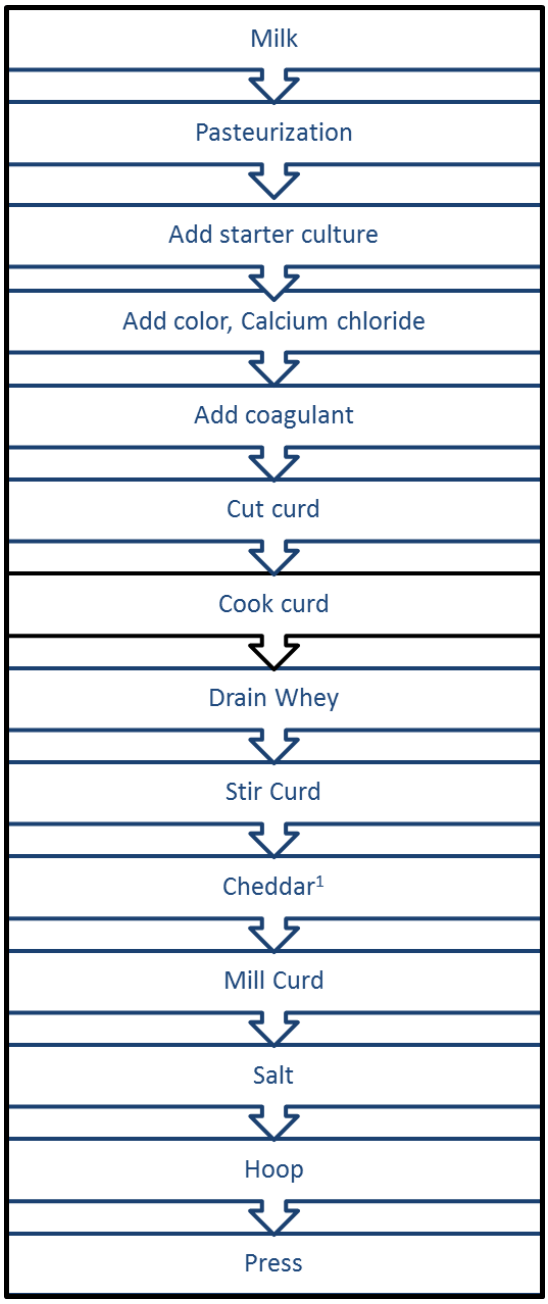

Figure 2: An outline of traditional Cheddar cheese manufacturing steps

1: Cheddaring omitted in stirred curd Cheddar cheese manufacture

2.4.4: Function of salt in cheese

It is well known that an excess consumption of sodium can cause negative health consequences. Before altering the sodium or seasoning profile of a food product, the functional properties of salt in foods must be considered. Perhaps the most immediately obvious function in cheese is that salt adds desirable flavor to the end product. The composition of the cheese is also affected by salt. The addition of salt decreases the water activity of the cheese (Fox et al., 2004). Therefore, removing salt would cause an increase in water activity of the cheese, and potentially create a more favorable environment in which pathogenic and non-pathogenic microorganisms could grow.

Salt is responsible for the control of lactic acid fermentation (through the control of starter and non-starter lactic acid bacteria), moisture level of the cheese, and protein texture, solubility, and conformation (Linklater, 1966; Fitzgerald and Buckley, 1985; Donnelly, 2004). Finally, salt contributes to the overall shelf life and safety of the cheese, through the inhibition of growth of spoilage organisms and foodborne pathogens.

During aging, salt has an effect on lipolysis—the breakdown of fatty acids within the cheese—a process that contributes to the flavor of cheese. Both starter lactic acid bacteria and non-starter lactic acid bacteria (NSLAB) control the extent of lipolysis. Lipases inherently present in milk and those contributed by NSLAB work to hydrolyze triacylglycerides present in cheese and release free fatty acids (FFA). The specific combination of FFA found in ripened Cheddar cheese varies from batch to batch, but most often contains short- and medium-chain fatty acids such as butyric, caproic,

caprylic, and capric. These FFA serve as important precursors in reactions that produce additional flavor compounds (Collins et al., 2003). When sodium is reduced, the overall rate of lipolysis is increased (Lindsay et al., 1982; Lesage et al., 1993). This is due to an increase in lipase production by NSLAB, which are inhibited by sodium (Lesage et al., 1993).

The effect of salt on lactic acid fermentation is highly correlated with the salt-in-moisture ratio (S/M). The S/M may be calculated by dividing the percentage of salt in cheese by the percentage of moisture. For example, a cheese with 2% salt and 38% moisture would have a S/M ratio of 5.26. A high quality Cheddar cheese usually has a S/M ratio between 4 and 6%. Turner and Thomas (1980) suggested that at a lower S/M ratio (~4%), *Lactococci* from starter cultures were able to utilize the majority of lactose in the cheese within a few days, while at a high S/M ratio these organisms were unable to metabolize lactose and as a result the concentration remained fairly high even after multiple weeks of storage. When this is the case, the residual lactose is then metabolized by NSLAB.

The addition of salt during manufacture results in the expulsion of moisture from the cheese (Linklater, 1966). As briefly mentioned, salt affects the development of the texture of Cheddar cheese. During aging, cheese undergoes proteolysis, a biochemical process responsible for modifications of cheese texture, and the formation of amino acids and peptide molecules which contribute to the end flavor of the cheese (Cruz et al., 2011). Initially, the casein proteins are hydrolyzed by enzymes naturally present in the cheese—most often plasmin—and any residual amounts of the coagulating enzyme used

during manufacture. As time goes on, further proteolysis is completed by non-starter lactic acid bacteria (NSLAB) (Donnelly, 2004). These organisms contribute protein decarboxylases and deaminases that further degrade the casein proteins. Because the salt in cheese controls the activity of NSLAB, as the concentration of salt is altered, the activity of the NSLAB is altered. When salt is removed, NSLAB activity increases, and as a result, the rate of proteolysis increases. This results in a softer, pastier texture and has been observed in several studies of Cheddar and other types of cheese (Thakur et al., 1975; Lindsay et al., 1982; Lesage et al., 1993).

Finally, sodium chloride has a preservative effect on the cheese, and contributes to the safety of the end product. The addition of sodium chloride lowers the water activity of the cheese, and thus lowers the ability of pathogens and undesirable microorganisms to grow. In combination with other intrinsic factors such as pH and a_w Cheddar cheese has often been considered an inhibitory environment for the growth of food pathogens. While there have been a few foodborne illness outbreaks associated with Cheddar cheese in the past few decades, these events were primarily a result of poor sanitization practices (Donnelly, 2004). Because of its typical intrinsic characteristics, when manufactured properly, Cheddar cheese is most often too harsh an environment to support foodborne pathogen growth.

Despite this fact, there have been studies of the behavior of pathogenic organisms in Cheddar cheese. Ryser and Marth (1987a) reported that when Cheddar cheese was manufactured with milk that had been inoculated with *L. monocytogenes*, the population gradually declined when the cheese was stored under refrigerated conditions. However,

when inoculated as a post-ageing contaminant, Shrestha et al. (2011) showed that *L. monocytogenes* was better able to survive in Cheddar cheese, in both full and lowered sodium products.

2.5: Low sodium Cheddar cheese

Requests for reduced sodium and low sodium food products have been in place for several decades (Lindsay et al., 1982; Fitzgerald and Buckley, 1985; Ryser and Marth, 1987a; Schroeder et al., 1988; Mehta, 1994; Aly, 1995; Ayyash and Shah, 2011). Often though, reduced sodium alternatives for products like Cheddar cheese are unable to match flavor and textural profiles of their full sodium counterparts and are ultimately deemed unacceptable by consumers (Johnson et al., 2009).

2.5.1: Composition and standard of identity

According to the FDA, in order for Cheddar cheese to be considered 'reduced sodium', or 'low sodium', it must comply with the standard of identity for Cheddar cheese, with one exception. The standard of identity states that reduced sodium and low sodium products must contain 25% less sodium than its conventional counterpart (of the same serving size), or no more than 280 mg/100g product, respectively (FDA, 2009). The composition of reduced or low sodium Cheddar cheese is very similar to that of its full sodium counterpart. Both start with the same basic ingredients—milk, cultures, enzymes, and salt—but additional salt replacers may be added to products from which sodium has been removed.

2.5.2: Processing and manufacture

Low sodium and reduced sodium Cheddar cheese are manufactured in much the same way as their full sodium equivalent. Some consideration may be given to the selection of starter cultures. Research has shown that certain starter cultures act at different rates in the presence of different cations. For example, *L. lactis* ssp. *lactis* displayed increased activity in the presence of potassium compared to the presence of sodium, and decreased activity in the combined presence of sodium and potassium (Garman et al., 1996). In the manufacture of cheeses in which sodium is lowered and possibly replaced, it is important to consider the effect of this reduction or replacement on the activity of the starter culture. The primary difference of course is in the amount of salt that is added during the salting step seen in figure 2. Based on the experience of the cheese maker, and targeted salt content, the appropriate amount of salt, and possibly, but not necessarily, salt replacement ingredient(s) are also added at this step.

2.6: Sodium reduction and salt replacement ingredients

Sodium reduction in foods is a current public health priority. The average sodium intake by most US residents substantially exceeds the recommended 2,300 mg/day (CDC, 2012a, c). Through an increase in reduced and low sodium food products, millions of cases of hypertension could be prevented, and billions of dollars in health care costs could be saved (Palar and Sturm, 2009; CDC, 2012a). Cheese is among the food products targeted for sodium reduction initiatives, as it is among the ten leading sodium contributing foods and accounts for nearly 4% of the sodium consumed in the average American diet (CDC, 2012c). However, reductions in sodium often result in flavor and

texture loss, and reduced acceptability. There are several strategies for the removal of sodium from processed food products, ranging from full replacement with a salt substitute, to a stealth-type of removal where sodium is slowly removed over time (IOM, 2010).

Past research indicates that it is possible to reduce sodium in Cheddar cheese (Lindsay et al., 1982; Reddy and Marth, 1993; Schroeder et al., 1988). While this research has continued recently (Shrestha et al., 2011), the study of sodium reduction from foods has expanded, and other types of cheese have also been studied. In an abstract submitted to the American Dairy Science Association, Ganesan et al. (2011) found that Mozzarella cheese with a 25% and 30% reduction in sodium had acceptable overall liking scores and similar functionality to full sodium pasta filata Mozzarella cheese. In a study of Halloumi—an unripened, brined cheese popular in Cyprus—Aayash and Shah (2011) found that a reduction in sodium and replacement by potassium chloride did not affect proteolysis in the cheese during brining. Ultimately, reductions in sodium may be made without compromising the quality of food products when sodium or salt replacement ingredients are used.

When reducing the sodium content in cheese, manufacturers must also consider the safety of the product. Salt in cheese is one of the main determinants of water activity. As such, it holds some control over microbial growth (Cruz et al., 2011). *L. monocytogenes* is a foodborne pathogen associated with dairy products and ready to eat foods, like Cheddar cheese. Although *L. monocytogenes* is already fairly salt resistant, it

is possible that a reduction in sodium could permit growth of the bacterium in products like cheese (Doyle and Glass, 2010).

2.6.1: Potassium chloride

Potassium chloride (KCl) is among the most common salt substitutes used in low sodium or reduced sodium meat products. In addition, it has been associated with decreased blood pressure (Aliño et al., 2010). In a study performed by Fitzgerald and Buckley (1985), it was found that a Cheddar cheese salted at a level of 1.6% with a 1:1 mixture (ionic strength basis) of sodium chloride and potassium chloride did not taste significantly different than Cheddar cheese salted with only sodium chloride. In addition, it was reported that Cheddar cheeses salted with KCl or a combination of KCl and NaCl did not have significantly different textural attributes when compared to the control Cheddar cheese (Fitzgerald and Buckley, 1985). The combination of KCl with NaCl has been used successfully in other products as well, indicating its potential to be used in Cheddar cheese (Gomes et al., 2011; Panagou et al., 2011). However, substitution of NaCl by KCl may result in an increase in water activity. This is because potassium has a higher molecular weight than sodium, so in an equivalent mass, there would be fewer molecules of potassium than sodium. With fewer molecules, KCl does not lower the water activity a_w to the same degree as NaCl (Grummer and Schoenfuss, 2011). It is important to note that KCl may be responsible for the contribution of a sour or bitter flavor that is experienced as the cheese ages, as well as a change in proteolysis (Cruz et al., 2011).

2.6.2: Magnesium chloride

Fitzgerald and Buckley (1985), found that after four months of ripening, Cheddar cheese salted with magnesium chloride, and Cheddar cheese salted with a mixture of magnesium chloride and sodium chloride scored significantly lower than Cheddar cheese salted exclusively with sodium chloride for attributes of flavor and texture. Cheeses salted with magnesium were said to have a bitter, metallic flavor (Fitzgerald and Buckley, 1985). Magnesium chloride has also been used in a salting system for Camembert cheese. However, it was found to increase lipolysis compared to control (full sodium) cheese. The lipolysis occurred at a faster rate than proteolysis in the cheese, and produced a significant bitter off-flavor (Lesage et al., 1993).

2.6.3: Calcium chloride

One study showed that after four months of ripening, Cheddar cheese salted with calcium chloride, and Cheddar cheese salted with a mixture of calcium chloride and sodium chloride scored significantly lower than Cheddar cheese salted exclusively with sodium chloride for attributes of flavor and texture (Fitzgerald and Buckley, 1985). Cheeses salted with calcium chloride were found to have a bitter, metallic flavor, or sometimes a notable sour flavor (Fitzgerald and Buckley, 1985; Cruz et al., 2011).

2.6.4: Other salt replacement ingredients: flavor enhancers

If potassium chloride, magnesium chloride or calcium chloride may not be used, it is possible that sodium reduction may be combined with the addition of other ingredients. Some companies have been observed using monosodium glutamate (MSG), a common flavor enhancer (Cruz et al., 2011). Other alternatives include yeast extract (YE),

hydrolyzed vegetable protein (HVP), disodium inosinate (IMP), and disodium guanylate (GMP). In a study of MSG, IMP and GMP, Giovanni and Guinard (2001) found that solutions made up of varying combinations of MSG and IMP or MSG and GMP produced a salty taste as intense as an NaCl solution, indicating the possibility of using these flavor enhancers in reduced sodium Cheddar cheese. Furthermore, this study suggested that the concentration of the flavor enhancers needed to exhibit the same intensity was much lower than the concentration of NaCl (5 mM or 10 mM versus 70 mM). Research on the use of these flavor enhancers in cheese is fairly limited, but the prospect of their use in low and reduced sodium cheese is exciting.

Chapter 3: Determining the effect of sodium reduction on the survival of *L. monocytogenes* and *B. anthracis* in Cheddar cheese

3.1: Introduction

To determine the effect of sodium reduction on the survival of *Listeria monocytogenes* and *Bacillus anthracis* spores in Cheddar cheese, in reduced and low sodium Cheddar cheese, a series of experiments were performed. In these experiments, Cheddar cheese with varying levels of sodium chloride reduction with and without replacement by potassium chloride (KCl) was manufactured and separately inoculated with a cocktail of these pathogens and stored at 4° or 12°C for 27-64 days.

3.2: Materials and methods

3.2.1: Cheese manufacture

Stirred curd Cheddar cheese was manufactured in duplicate cheese makes in the J. Warthesen Food Processing pilot plant located at the University of Minnesota. The schedule used in each cheesmake of two batches of Cheddar cheese is shown in table 2, which also includes the targeted temperature, pH and titratable acidity (TA) at each step. These parameters, especially TA are strong indicators of the activity of the starter culture during cheese manufacture.

Table 2: Typical manufacturing scheme for stirred curd Cheddar cheese

Step	Time (h)	Target temp. (°C)	Target pH	Target TA
Starter added	0	31.1	N/A	0.16
Color & $CaCl_2$ added	0.5	31.1	N/A	
Rennet added	1	31.1	Drop of 0.1 from start	0.17
Cut coagulum	1.5	31.1	N/A	0.10
Agitate curd	1.75-2.25	Gradual increase from 31.1 – 38.9	N/A	0.10
	2.25-3	Continue to maintain temp.	NA	0.10-0.12
Drain whey	3-4	38.9	N/A	N/A
Stir curd	4	31.1	N/A	Increase from 0.12-0.17
Salt	4.5	31.1	N/A	0.24
Hoop	5	31.1	N/A	N/A
Press	5.25-21.25	N/A	N/A	N/A

N/A = not applicable

In each experimental batch, milk was pasteurized at 73.3°C for 20 s on a plate and frame-style milk pasteurizer, divided into two stainless steel open vats with automatic agitation (Kusel Equipment Company, Watertown, WI) at 31.1°C, and separately inoculated with one of two single-strain starter cultures: *Lactococcus lactis* ssp. *lactis* (Direct vat set, trade name M58, CHR Hansen, Milwaukee, WI), or *Lactococcus lactis* ssp. *cremoris* (Bulk set, trade name D71, CHR Hansen) at a level of 0.01% and 1% of the weight of the milk, respectively. Direct vat set cultures are typically used at markedly lower concentrations than traditional bulk cultures. After 30 minutes to one hour of heating (depending on the TA which was used to reflect the activity of the starter culture), annatto color (AFC W/S 1x 70463, CHR Hansen) and calcium chloride (CAL-SOL 71257, Chr. Hansen) were added at a level of 0.0066% and 0.02% (wt/wt) of the

starting weight of milk, respectively. Color and calcium chloride were stirred into heated milk for 30 minutes, before adding the coagulating enzyme chymosin at a level of 0.01% of the starting weight of milk (2X Chymax CHYE-002 CHR Hansen). A gel was allowed to form over the course of 30 min before cutting the coagulum into curds, using 9 mm harps (commonly referred to as a 3/8 inch knife).

The cut curds were allowed to heal for approximately 5 minutes, and were agitated in the whey as the temperature was gradually increased from 31.1°C to 38.9°C for 30 min. It was crucial for this increase to happen gradually so that the appropriate texture could form. After the temperature had reached the cooking temperature, the curds were stirred for an additional 45 min. Once cooking was complete, the whey was drained (while agitation continued) in three parts during the course of 70 min. The first drain emptied 20% of the whey from each vat. Approximately 40 min after the first drain, another 20% of the whey was drained from each vat. Approximately 30 min after the second drain, the remaining whey was drained from each vat. Once all of the whey had been drained, the curds were agitated until the TA reached 0.24 or 0.25.

After the appropriate TA developed, five units of 13.6 kg of curd were placed into separate salting bins with drain holes where they were treated with the appropriate salting system outlined in table 3. The amount of sodium chloride (NaCl) (Top-Flo® Evaporated Salt, Cargill, Inc., Minneapolis, MN), and potassium chloride (KCl) (Premier™ Potassium Chloride 8799, Cargill, Inc.) used in each treatment was based on the full NaCl level traditionally used in cheese made in the University of Minnesota pilot plant, and appropriate levels of KCl needed to keep the same a_w of the cheese in all

treatments (targeted a_w was 0.97). Salting was performed by hand, in three equal applications in five min intervals. The curds were stirred by hand to ensure a homogenous distribution of salt and/or KCl throughout the entire mass of cheese.

When salting was complete, the curds were packed into 9.1 kg Wilson-style hoops, and pressed overnight at 276 kPa. After pressing, the blocks of cheese were removed from the hoops and vacuum sealed (Multivac Vacuum Packager, Koch, Kansas City, MO) and stored at 4-5°C for one week before chemical and microbiological testing could begin.

Table 3: Salt treatments used in the manufacture of stirred-curd Cheddar

Treatment	NaCl added (g/kg curd)	KCl added (g/kg curd)
Full sodium	29.04	0.00
25% Reduced sodium	21.78	0.00
25% Reduced sodium with KCl	21.78	9.26
55% Reduced sodium	13.07	0.00
55% Reduced sodium with KCl	13.07	20.38

After cheese was manufactured and stored for one week, it was prepared for further testing. Each block of cheese was cut into 8 equal subsections. From these subsections, two were randomly chosen as replicates for chemical and microbiological testing. These blocks were shredded separately in a food processor (Cuisinart DFP-14BCN, Cuisinart, East Windsor, NJ) and then used for chemical and microbiological testing.

3.2.2: Chemical analysis of cheese

Titratable acidity

Titratable acidity was measured during cheese manufacture to monitor the acidity level of the cheese as a reflection of starter culture activity. To determine the titratable acidity, two drops of phenolphthalein indicator were added to 9.00 g milk or whey as appropriate. Sodium hydroxide (NaOH, 0.1 N) was added drop-wise to the milk or whey sample from a burette standardized for TA measurement. Once the phenolphthalein reaction was complete (sample turned a faint pink color), the volume of NaOH that had been dispensed was read corresponding to the TA.

Moisture

Moisture analysis was completed in a vacuum oven according to The Standard Methods for the Evaluation of Dairy Products ("standard methods") (Marshall, 1992). Disposable aluminum moisture dishes were labeled and dried in a forced draft oven (Lab-Line Imperial II, Thermo Fisher Scientific, Waltham, MA) for 3 h with temperature maintained at 100°C. After cooling in a dessicator and weighing the pans, 3 ± 0.5 g shredded cheese was weighed into each pan, in duplicate for each replicate of each treatment. Pans were then placed into the vacuum oven (Napco 5851, Thermo Fisher Scientific) for 5 h. The conditions of the oven were maintained at 100°C, 25.5 inches of mercury and an air flow rate of 117 mL/min. Finally, the aluminum dishes were allowed to come to room temperature in a desiccator for 30 min before they were weighed. Percent moisture was calculated as:

$$Percent\ moisture = \frac{Loss\ of\ mass\ during\ drying}{Weight\ of\ cheese\ sample} \times 100$$

Moisture of the cheese was determined after initial manufacture, and after 1 and 2 months of storage at 4°C.

pH

The pH of the cheese was measured in duplicate for each replicate from each treatment, initially after manufacture, and periodically during refrigerated storage over the course of nine months. Analysis of pH was performed according to standard methods (Marshall, 1992). The pH meter (Oakton pH meter 510 series, Oakton Instruments, Vernon Hills, IL) was calibrated before each use, and pH was measured by fully submerging the probe into shredded cheese.

Water activity (a_w)

The a_w of cheese was measured in duplicate for each replicate from each treatment using a Decagon pa_w kit and water activity meter (Decagon Instruments, Inc., Pullman, WA) initially after manufacture, and periodically during refrigerated storage. Following kit instructions, the a_w meter was calibrated before each use using standards with a_w of 0.76 and 0.25. Each measurement of a_w required 1 to 2 g shredded cheese that had been manipulated to fully cover the bottom of a sample cup.

Protein

The amount of protein in the cheese was determined immediately after manufacture through the analysis of nitrogen content, based on the Dumas method. Cheese samples were analyzed by the Leco TruSpec® N (Leco Corporation, St. Joseph, MI). For analysis, 0.15 g cheese was weighed accurately and placed into a gelatin capsule (Leco Corporation) that was loaded into the instrument carousel. After

performing necessary calibration steps with a glycine standard (Leco Corporation), analysis was performed in duplicate for each replicate from each treatment. The protein factor used was 6.38.

Fat

Analysis of fat was performed in duplicate for each replicate from each treatment after manufacturing. Fat analysis was completed using the Babcock method according to standard methods (Marshall, 1992). First, 9 g of cheese was accurately weighed into a Paley bottle, to which 10 mL of hot water (60°C) was added. The bottle was swirled by hand to suspend the cheese within the water and bottles were allowed to cool to room temperature. While the bottle was held at a slight angle, 15 mL of sulfuric acid (specific gravity 1.825, RICCA Chemical Company, Arlington, TX) was added in three additions: 8, 4 and 3 mL while swirling by hand in between additions and completing all additions within 20 s. After all of the acid was added, bottles were swirled by hand until no large particles remained, and were then placed on a mechanical shaker for 5 min at moderate speed (Lab-Line Rotator, Thermo Fisher Scientific, Waltham, MA).

The bottles were then placed into a centrifuge (The Improved Jalco, Jalco Motor Company, Union City, IN), and centrifuged for 5 minutes. After 5 minutes, the centrifuge was stopped and hot water (60°C) was added until the bulb of each bottle was filled to within 0.6 cm of the base of the neck. Bottles were then centrifuged for 2 minutes, after which hot water was added until the liquid fat column reached the top graduation of the scale on the bottle neck. Bottles were then centrifuged for 1 min. After centrifugation was complete, bottles were transferred to a 57.5°C water bath (VWR Scientific 1245 PC, VWR International, Radnor, PA) to temper for 5 min. Fat columns

were read to the nearest 0.5% using a standardized caliper immediately after tempering. Readings were taken from the bottom of the lower meniscus to the top of the upper meniscus. Any samples with milky, cloudy or charred fat columns were rejected and reanalyzed.

Ash

Analysis of ash was performed in duplicate for each replicate from each treatment immediately after manufacture, following standard methods. Labeled porcelain crucibles were dried in a forced air oven (Lab-line Imperial II, Thermo Fisher Scientific) at 100°C for one hour. Crucibles were then cooled to room temperature in a desiccator. After cooling, 2 g of cheese was weighed into each crucible, and samples were evaporated to dryness in a forced air oven (1 h at 100°C) (Lab-line Imperial II, Thermo Fisher Scientific).

Crucibles were transferred into a safety cabinet with hood, where they were placed over a Bunsen burner and slowly carbonized. Samples were then transferred to a muffle furnace (Thermolyne, Sybron Corporation, Thermo Fisher Scientific) where they were incinerated at 550°C for 12 h (Dubuque III Solid State Controller, Dubuque Corporation, Dubuque, IA). Samples were allowed to cool in the muffle furnace for 8 h after incineration before being cooled to room temperature in a desiccator. Percent ash was calculated using the following equation:

$$Percent\ ash = \frac{Weight\ of\ residue}{Weight\ of\ sample} \times 100$$

Sodium and potassium

Sodium and potassium content were determined through the use of atomic absorption of the ashed cheese samples. Analysis was completed in duplicate for each replicate from each treatment. The method used for atomic absorption was adapted from the International Organization for Standardization (ISO) and published methods (Schroeder et al., 1988; Mehta, 1994; Grummer and Schoenfuss, 2011). Ash was suspended in 1 mL of 0.1 N trace element free nitric acid. The contents of the crucible were quantitatively transferred into a 250 mL volumetric flask. The crucible was rinsed three times using double distilled (DD) water, and the contents from each rinsing were also transferred to the volumetric flask. Finally, the flask was brought to volume with DD water at room temperature. Each sample was then subjected to a 10-fold dilution before being transferred to a conical centrifuge tube before sampling.

Standards were created by diluting 1000 mg/L sodium and potassium solutions (Thermo Fisher Scientific, Fair Lawn, NJ) for development of standard curves with solutions diluted to 7.5, 5.0, 3.0, 2.5, 1.0, 0.5, and 0.05 mg/L. In order to maintain continuity between the standard curve and cheese samples, the standard solutions were diluted using a solution of 1 mL of 0.1 N trace element free nitric acid brought to volume in a 250 mL volumetric flask with DD water. Samples were analyzed via atomic absorption using a Perkin Elmer AAnalyst spectrometer 100 (Perkin Elmer, Inc., Waltham, MA) with compressed air as the oxidant, and acetylene gas as the fuel. Samples were analyzed using a Na-K cathode lamp under the following conditions: wavelength = 589 nm; slit width = 0.2 for Na, 0.7 for K; read time = 5 s; read delay = 2 s, oxid flow = 10, fuel flow = 3, re-slope = 2.5 mg/L.

3.2.3: Bacterial strains and inoculation of cheese

Listeria monocytogenes

The strains of *L. monocytogenes* that were used to inoculate cheese before performing microbiological analysis are listed in table 4.

Table 4: *Listeria monocytogenes* strains used to inoculate Cheddar cheese samples

Strain	Ribotype	Serotype	Source
J1-119	1038B	4b	Human, Mexican-style cheese outbreak, CDC 1985
N1-227	1044A	4b	Hot dog, outbreak CDC 1998
N3-031	1053A	1/2a	Hot dog, sporadic case WHO 1989
R2-500	1042B	4b	Mexican-style cheese, outbreak CDC 2000
R2-502	1051B	1/2b	Chocolate milk, outbreak CDC 1994

These strains were obtained from frozen cultures in the Food Safety Microbiology Laboratory at the University of Minnesota, in St. Paul, MN. These strains were originally provided by Dr. Martin Wiedmann and are part of the International Life Sciences Institute collection (Gadotti, 2011). Frozen stock cultures are maintained in glycerol at -55°C. To make a working culture, frozen cultures were first streaked individually on to tryptic soy agar plates (TSA) (Neogen Corporation, Lansing, MI) that were incubated for 24 h at 37°C. One colony from each plate was picked and used to inoculate a TSA slant that was held at 4°C for up to one month at which time a replacement slant was made. To prepare overnight cultures, colonies from the TSA slant were used to inoculate 9-mL tryptic soy broth (TSB) (Neogen Corporation) tubes that were incubated at 37°C for 24 h. The overnight cultures were used to inoculate cheese samples.

Bacillus anthracis

The strains of *Bacillus anthracis* used to inoculate Cheddar cheese were 7702p610 and UT 258. Both are derivatives from Sterne strain, carry erythromycin resistance, and produce green fluorescence protein. Strains were obtained from frozen cultures in the Food Safety Microbiology Laboratory at the University of Minnesota. The strains were originally provided by Theresa Kohler at the University of Texas-Houston Medical School (Leishman, 2009). While unique because of the presence of green fluorescence protein and a resistance to erythromycin, these strains were also chosen because they had been cured of their pXO2 plasmids, rendering them avirulent. Stock cultures are maintained in glycerol at -55°C.

To prepare a working spore suspension, a small amount of each frozen stock culture was transferred into a tube with 9 mL TSB and incubated at 37°C for 18 h. Using the spread plate technique, 0.1 mL of each overnight culture was dispensed onto a plate of new sporulation media (NSM) (Neogen Corporation) and incubated at 37°C for 48 h, followed by 24 h incubation at 23°C. The biomass from each plate was then re-suspended in 30 mL sterile water, transferred to a centrifuge tube, and incubated at 22°C for 72 h. Finally, the spores were harvested through centrifugation (Sorvall RC-5 Superspeed Refrigerated Centrifuge, Dupont Company, Wilmington, DE) at 8,000 x g at 4°C. Centrifugation was followed by spore washing, repeated in triplicate. The concentration of spores was verified to be greater than 95% spores through phase-contrast microscopy. Spore suspensions were stored at 4°C for no more than 2 months.

Inoculation of cheese

To perform time-course testing, shredded cheese was inoculated with overnight cultures or viable spore suspensions and then divided into individual 10 g sampling units that could be vacuum sealed and stored for the duration of time-course testing.

To inoculate cheese with *Listeria monocytogenes*, after the preparation of overnight cultures, 2 mL from each strain were added to sterile test tubes, and vortex mixed for 30 s. Serial dilutions were performed to reach an appropriate concentration of cells of 10^4 CFU/g, and 1 mL of *L. monocytogenes* cocktail was added to a small plastic bag containing 99 g of full sodium Cheddar cheese. The bag was held closed, and the contents of the bag were manually agitated for 90 s to ensure adequate mixing and homogeneous distribution of the inoculum. The inoculated cheese was distributed in 10 g portions into an appropriate number of labeled bags depending on the number of samples taken over the duration of time-course testing. This process was repeated for the remaining cheese treatments. Additionally, a corresponding number of uninoculated control samples were prepared for each treatment based on the number of samples taken over the duration of time-course testing. Bags were vacuum sealed and stored at 4°C or 12°C depending on the phase of testing.

Cheese was inoculated with *B. anthracis* spores. To ensure that the cheese was inoculated with only viable spores, and not vegetative cells, suspensions were heated at 75°C for 30 min (Xu et al., 2006). After heat-shocking the spore suspensions, 5 mL aliquots from each strain were mixed in a sterile test tube for 30 s. A volume of 1 mL of the spore cocktail were added to a small plastic bag containing 99 g of full sodium Cheddar cheese. The bag was held closed, and the contents of the bag were manually

agitated for 90 s to ensure adequate mixing and homogeneous distribution of the inoculum. The inoculated cheese was distributed in 10 g portions into an appropriate number of labeled bags depending on the number of samples taken over the duration of time-course testing. This process was repeated for the remaining treatments. Bags were vacuum sealed and stored at 4°C or 12°C depending on the phase of testing. Negative (uninoculated) controls were also prepared for each treatment and sampling occasion.

3.2.3: Experimental design

Stirred curd Cheddar cheese was manufactured in duplicate batches and used throughout the duration of this project. Three major experimental stages were conducted during the maturation of cheese (Figure 3). At the beginning of each stage, replicate samples of cheese from each batch were inoculated as described above. Inoculated samples were stored at 4° and 12°C.

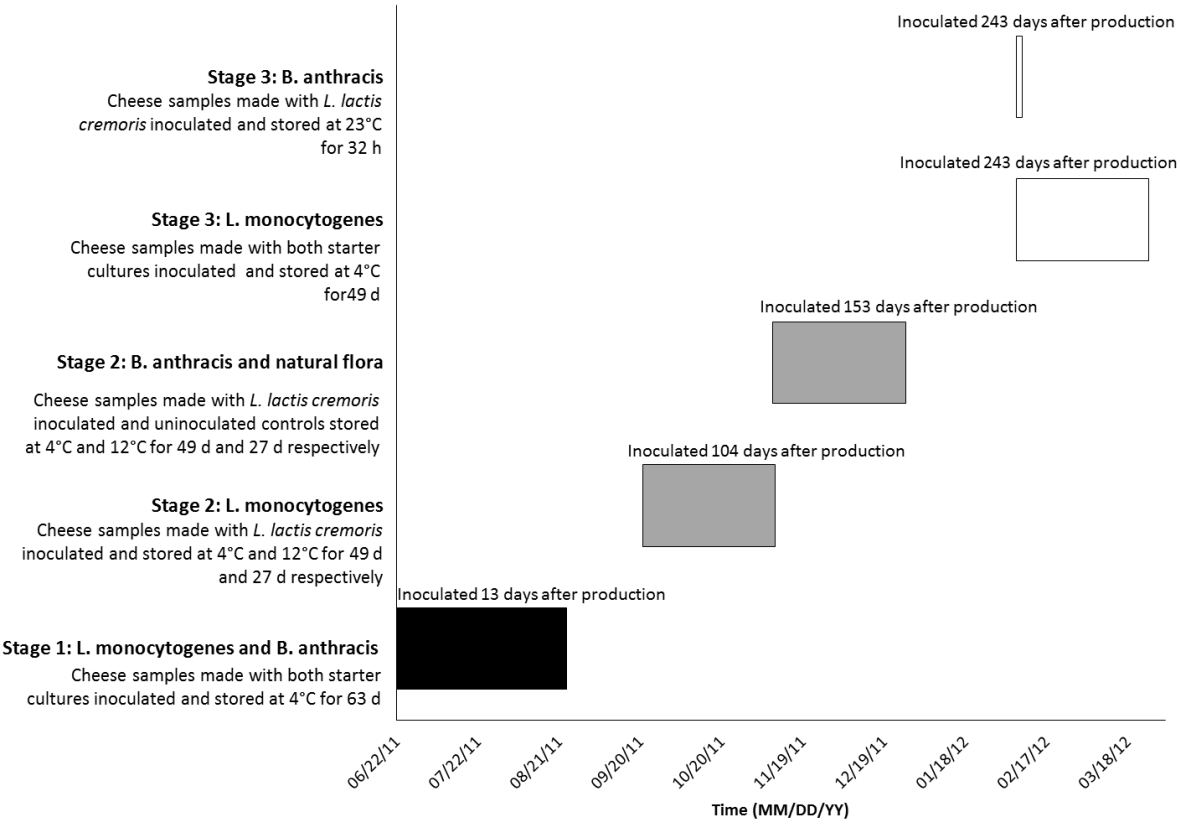

Figure 3: Stages of microbiological testing performed when determining the effect of sodium reduction on the survival of *L. monocytogenes* and *B. anthracis* in Cheddar cheese.

In stage 1, cheese made using both starter cultures was inoculated with each pathogenic bacterium and stored at 4°C for 63 days. Near the end of stage 1, counts of *L. monocytogenes* were below the limit of detection; to obtain additional data, selective enrichment methods from literature were used (Schamberger et al., 2004): cheese samples were enriched with UVM 1 *Listeria* enrichment broth (Neogen) for 24 h at 37°C. After enrichment, samples were processed as previously described. Using 5 CFU/g as

the theoretical limit of detection, positive samples obtained by enrichment only were assigned a count of 0.3 log CFU/g to be used in calculating the population of *L. monocytogenes* from those samples.

In stage 2, only cheese made with *L.* lactis ssp. *cremoris* as the starter culture was inoculated with each bacterium; it was stored at 4° and 12°C for 49 and 27 days respectively. In stage 3, cheese made with both starter cultures was inoculated with *L. monocytogenes* and kept at 4°C for 49 days. Cheese made with *L. lactis* ssp. *cremoris* was inoculated with *B. anthracis* spores and incubated at 23°C for 32 h. Most of the samples stored at 4°C were tested every 7 days, and all samples kept at 12°C were analyzed every 3 days. At 23°C, samples were tested every 8 hours.

For each sampling occasion, cheese samples were removed from storage and homogenized (Stomacher Lab Blender 4000, Seward Laboratory Systems, Inc., Port Saint Lucie, FL) with 90 mL buffered peptone water (BPW) (Neogen Corporation,) for 2 min. In stage 2, after homogenization and appropriate 10-fold serial dilutions, controls prepared with uninoculated cheese were spread plated on plate count agar (PCA) (Neogen Corporation) to enumerate total plate count, and de Man, Rogosa, and Sharpe agar (MRS) (Neogen Corporation) to enumerate lactic acid bacteria. Samples inoculated with *L. monocytogenes* were serially diluted and spread plated on PALCAM agar (Neogen Corporation). Samples inoculated with *B. anthracis* were serially diluted and spread plated on TSA supplemented with 50 μg/mL erythromycin, (ERM-TSA) as recommended by Leishman (2009). To obtain counts of natural spore forming organisms and *B. anthracis* spores, serially diluted negative controls and samples inoculated with *B.*

anthracis were heat shocked for 30 minutes in a water bath set at 75°C and spread plated on TSA and ERM-TSA respectively. All plates were incubated at 37°C for 24-48 h. All plating was performed in duplicate for each replicate from each treatment. Population counts were transformed to log CFU/g of *L. monocytogenes* or *B. anthracis* accordingly.

3.2.4: Statistical analysis

Upon completion of data collection, the data were analyzed using the MIXED PROCEDURE model in SAS computer software, version 9.2 (SAS Institute, Inc., Cary, NC). Fisher's least significant difference was used as a summary statistic.

3.3: Results

3.3.1: Chemical analysis of Cheddar cheese, reduced, and low sodium Cheddar cheese

There were no differences in gross composition based on the starter culture used to manufacture the cheese, nor in the salt treatment applied to the cheese (Table 5). The mean ash, sodium, and potassium contents varied based on salt treatment, as expected.

Table 5: Chemical analysis of Cheddar cheese, reduced, and low sodium Cheddar cheese

Salt Treatment	*Fat (%)*	*Moisture (%)*	*Protein (%)*	*Ash (%)*	*Sodium (mg/100g)*	*Potassium (mg/100g)*
Control[1]	32.5[a]	38.45[a]	23.87[a]	3.31[a]	501[a]	36[a]
-25% salt[1]	33.0[a]	37.20[a]	24.38[a]	2.87[b]	393[b]	33[a]
-25% salt + KCl[1]	33.0[a]	37.11[a]	24.72[a]	3.48[c]	441[b]	292[b]
-55% salt[1]	33.0[a]	37.01[a]	24.08[a]	2.39[d]	201[c]	29[a]
-55% salt + KCl[1]	33.0[a]	37.40[a]	24.91[a]	3.57[e]	251[c]	576[c]
Control[2]	32.5[a]	38.92[a]	24.05[a]	3.34[a]	488[a]	31[a]
-25% salt[2]	32.5[a]	39.02[a]	24.39[a]	3.07[b]	432[b]	32[a]
-25% salt + KCl[2]	32.0[a]	38.57[a]	24.36[a]	3.45[c]	415[b]	305[b]
-55% salt[2]	33.0[a]	37.86[a]	24.02[a]	2.49[d]	264[c]	36[a]
-55% salt + KCl[2]	32.0[a]	38.45[a]	24.76[a]	3.68[e]	273[c]	644[c]
Std. deviation	0.7	1.08	0.87	0.45	111	233

a-e: Mean values within the same column with the same letter superscript are not significantly different. No difference in gross composition based on starter culture was observed; the same superscripts apply to measurements of attributes obtained from samples of cheese made using both starter cultures.

1: Cheese made with the starter culture *L. lactis* ssp. *lactis*
2: Cheese made with the starter culture *L. lactis* ssp. *cremoris*

While analyzed initially after manufacture, moisture analysis was also performed after 1, 2 and 3 months of storage at 4°C. During storage, the moisture content of cheese made with all salt treatments of cheese decreased slightly. The difference in moisture at each time period was not significant between starter cultures (Figures 4 and 5). After 85 days of storage at 4°C, there were slight differences between the moisture contents of cheese manufactured using the starter culture *L. lactis lactis*. There was no difference in moisture contents between cheeses with and without potassium chloride at the same sodium reduction level (Figure 4).

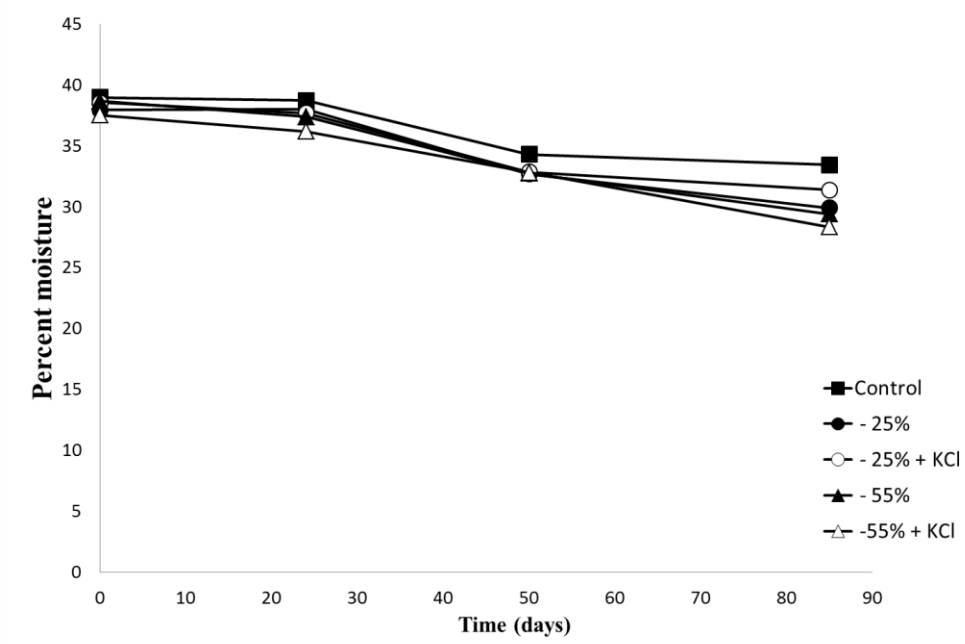

Figure 4: The effect of sodium reduction on the moisture content of stirred curd Cheddar cheese manufactured with *L. lactis* ssp. *lactis* starter culture. Time 0 denotes 13 days after production. Error bars represent standard error; each data point represents the average of 8 measurements.

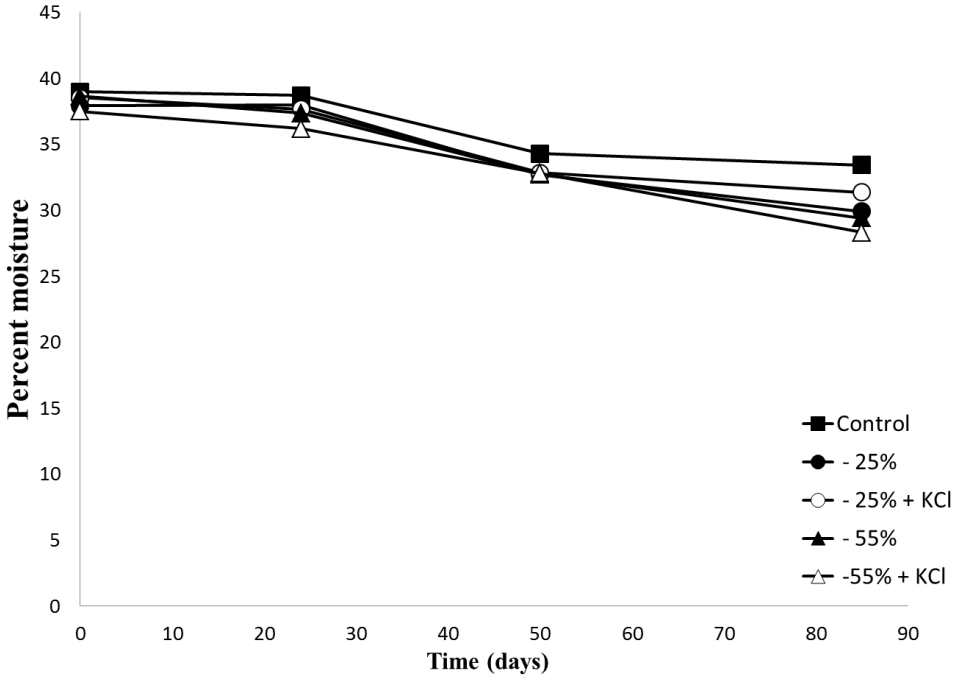

Figure 5: The effect of sodium reduction on the moisture content of stirred curd Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* starter culture. Time 0 denotes 13 days after production. Error bars represent standard error; each data point represents the average of 8 measurements.

In addition to moisture, water activity was also monitored over the course of 85 days of storage. A measurement of the initial water activity was taken, but was discarded due to technical issues. During the 60 days after this measurement, the water activity decreased slightly from an average initial measurement of 0.94 to 0.93 (Figures 6 and 7). At any given time point, there was no difference in water activity based on starter culture or salt treatment of the cheese.

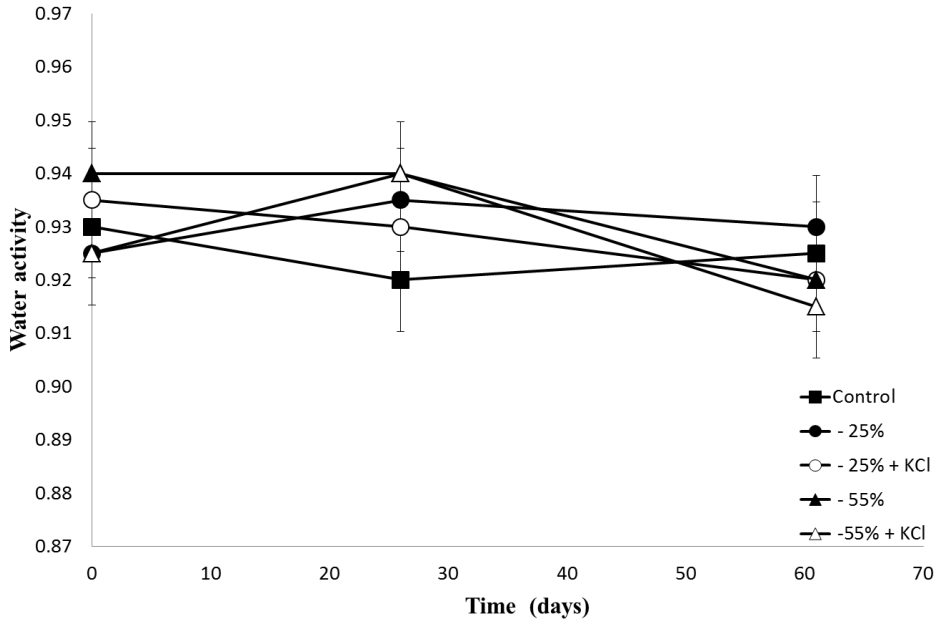

Figure 6: The effect of sodium reduction on the water activity of stirred curd Cheddar cheese manufactured with *L. lactis* ssp. *lactis* starter culture. Time 0 denotes 37 days after manufacture. Error bars represent the standard error; each data point is an average of 8 measurements.

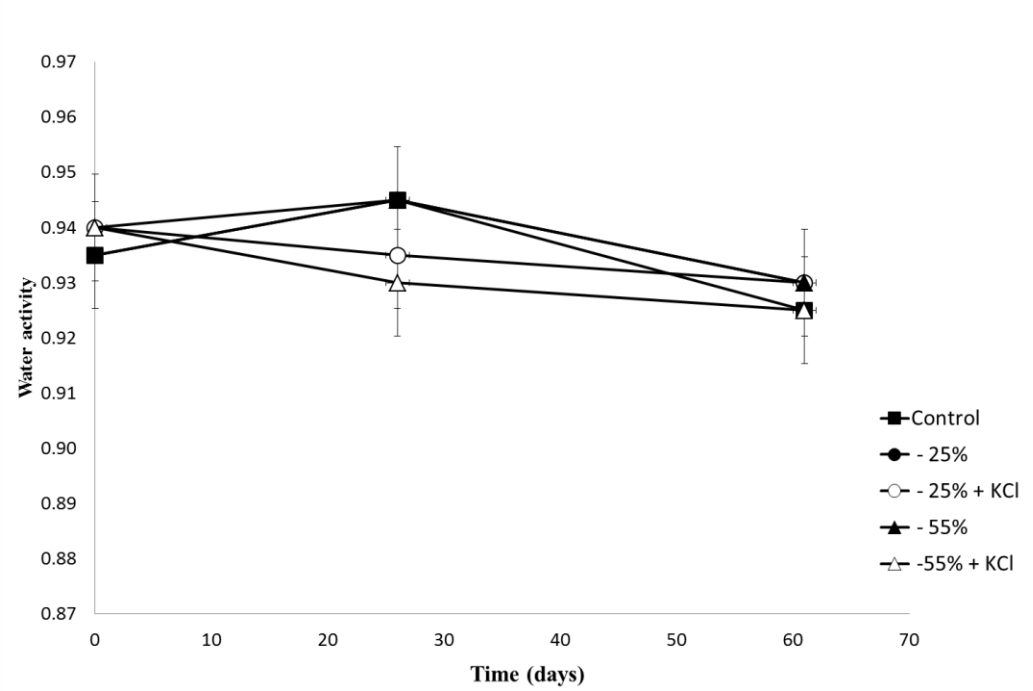

Figure 7: The effect of sodium reduction on the water activity of stirred curd Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* starter culture. Time 0 denotes 37 days after production. Error bars represent standard error; each data point is the average of 8 measurements.

Finally, pH was monitored over the course of nine months of storage. Figure 8 shows the combined results of pH from samples made with both starter cultures. During this time, the average pH of stirred curd Cheddar cheese increased gradually from an initial pH of 4.65 to a final pH of 5.3. No difference in pH due to starter culture used in manufacture, or salt treatment of the cheese at any given testing event was observed.

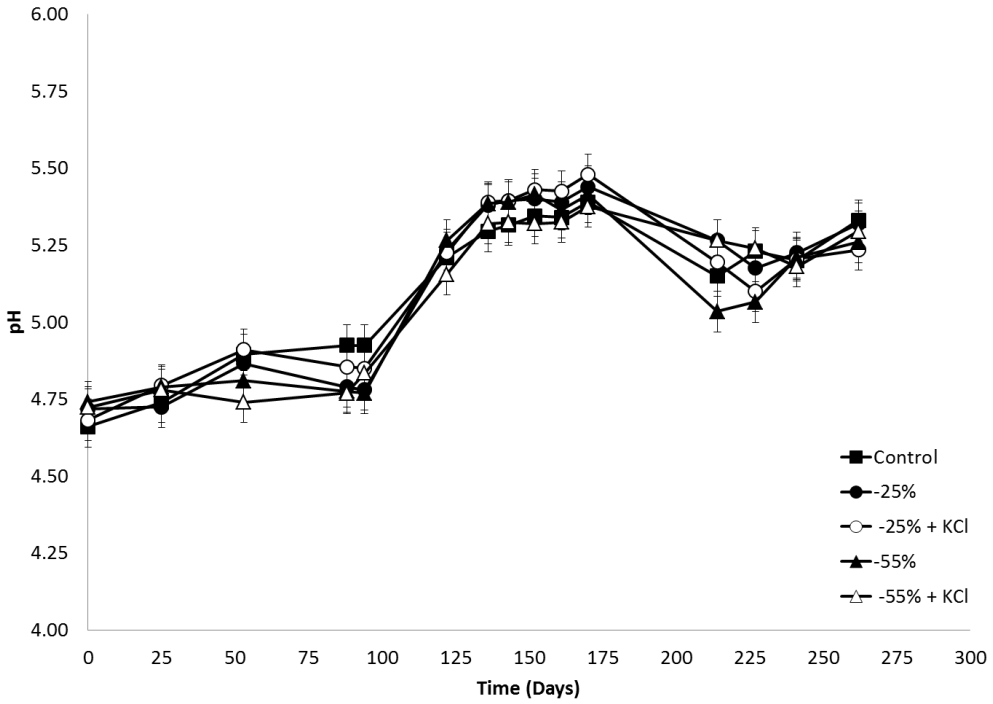

Figure 8: The effect of sodium reduction on the pH of Cheddar cheese. Time 0 denotes 13 days after production. Error bars represent standard error; each data point represents the average pH of cheese samples made from both starter cultures.

3.3.2: Natural microorganisms in Cheddar cheese, reduced and low sodium Cheddar cheese

While physical and chemical data may be used to determine the potential for growth of food pathogens, monitoring the natural background flora of the cheese was also completed. Enumeration of natural background flora was not completed until the second stage of the experimental design. The cheese was allowed to age 5 months before the count of natural microorganisms testing took place. By this time, it had been determined that all results from the first stage of testing revealed no differences in measurements

based on starter culture, so only cheese manufactured using the starter culture *L. lactis* ssp. *cremoris* was tested in the enumeration of background flora. Furthermore, in the second stage of microbiological testing, the cheese was stored at both 4° and 12°C to determine whether the temperature of storage had an effect on survival of natural organisms present in cheese.

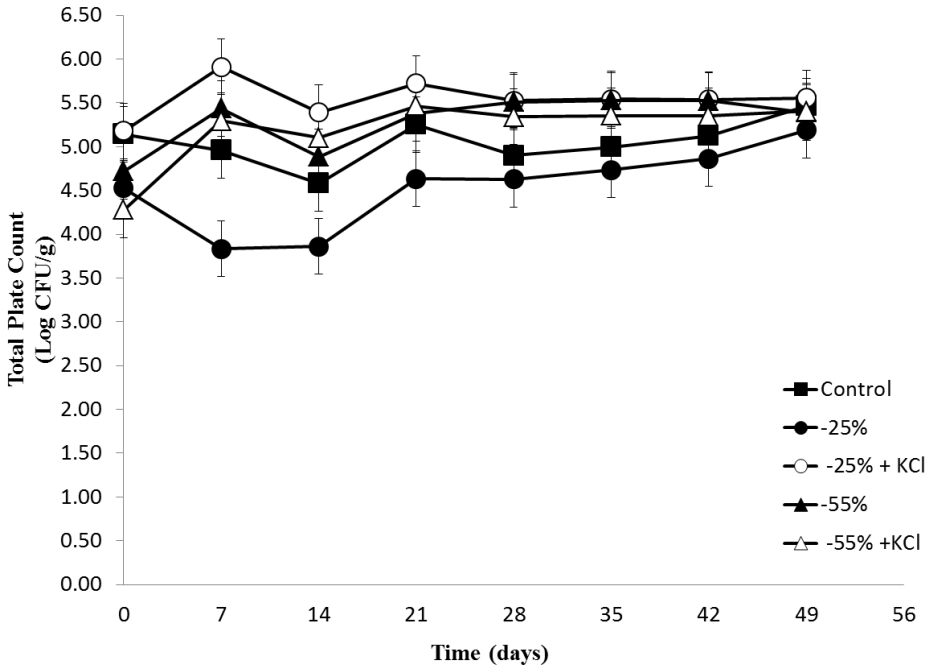

Figure 9: Total plate count of Cheddar cheese made using *L. lactis* ssp. *cremoris* and stored at 4°C.

Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

Cheese stored at 4°C had an initial total aerobic population that ranged from 4.25 to 5.25 log CFU/g. At day 7 and day 14, the 25% reduced sodium treatment samples had significantly lower aerobic plate counts (APC) than the control and the other treatments,

with a population around 4 log CFU/g. However, those were the only data points in which any difference based on salt treatment was observed (Figure 9). In cheese stored at 12°C, the initial APC was approximately 5.5 log CFU/g in all treatments. During the first 14 days there were some consistent increases in populations, but for most of the reminder of the experiment, APC values were very similar to initials. Overall there was no observed difference based on salt treatment at any given time point (Figure 10).

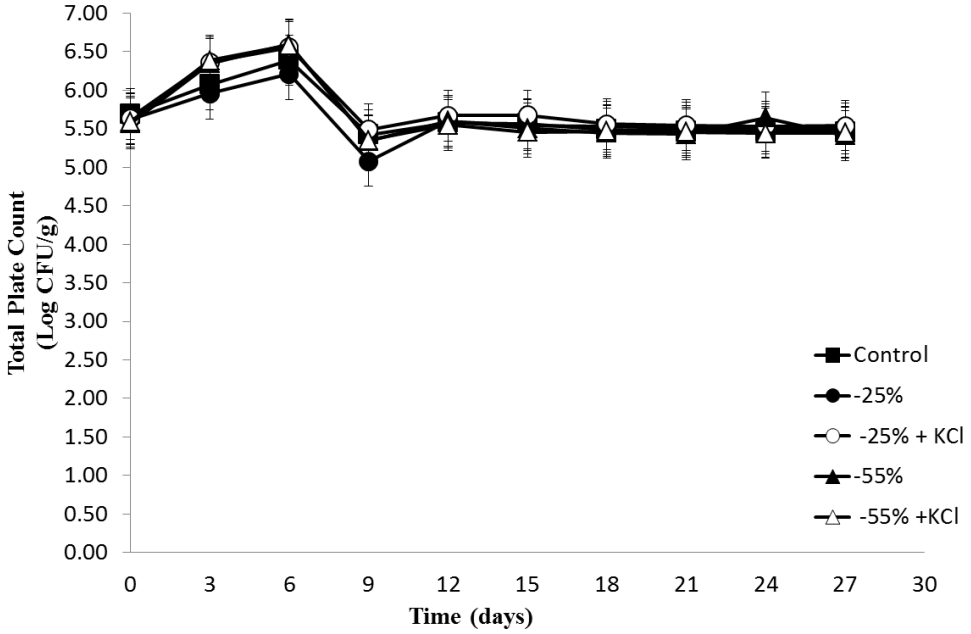

Figure 10: Total plate count of Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 12°C.

Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

Lactic acid bacteria are naturally present in milk and as such often found in cheese. The total population of lactic acid bacteria of the cheese was measured over the same period of storage as the aerobic plate count.

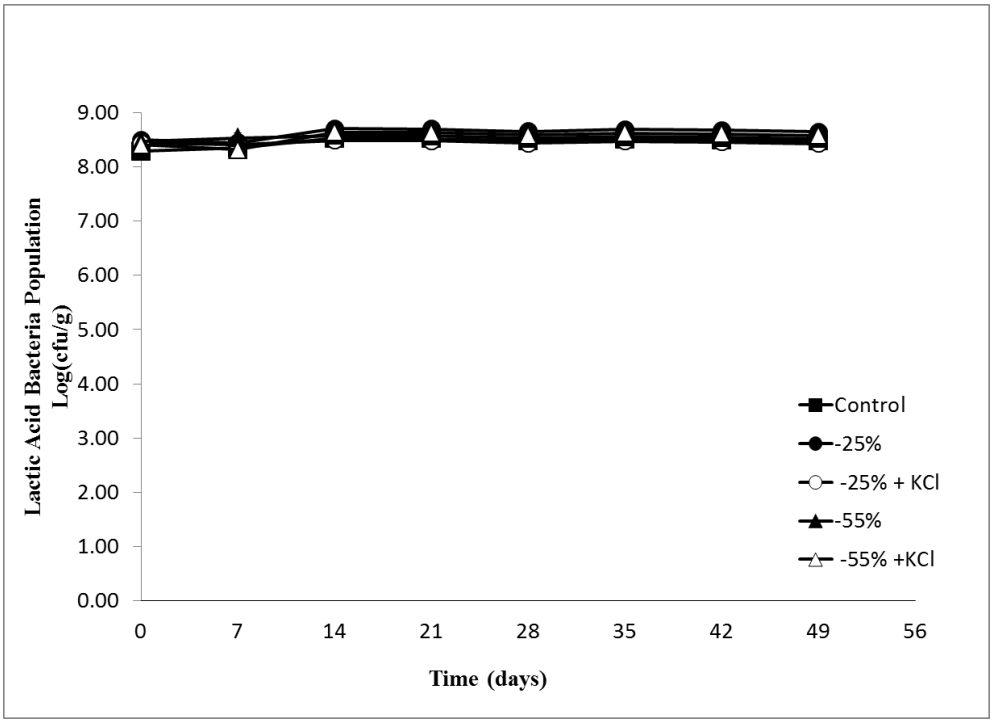

Figure 11: The effect of sodium reduction on the lactic acid bacteria population of Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 4°C. Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

The total population of lactic acid bacteria remained constant at 8.5 log CFU/g in cheese stored at both 4°C and 12°C. In cheese stored at both temperatures there was no difference in lactic acid bacteria population based on salt treatment at any given testing

occasion. Further analysis revealed that there was no difference in lactic acid bacteria population based on storage temperature (Figures 11 and 12).

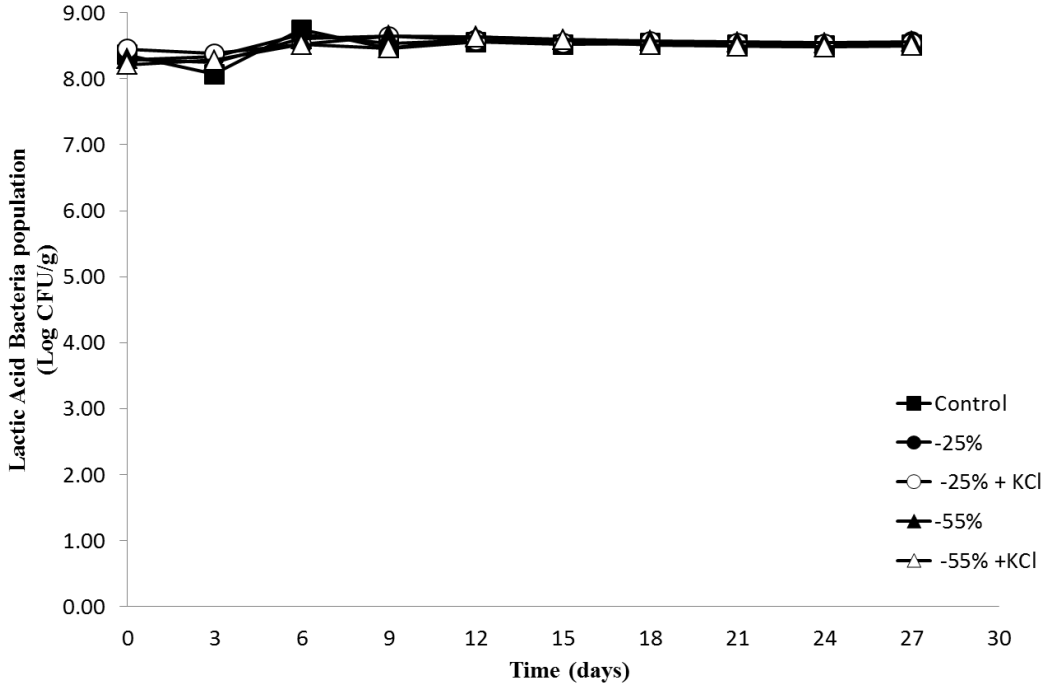

Figure 12: The effect of sodium reduction on the lactic acid bacteria population of Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 12°C. Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

The third natural microbiological population enumerated was that of aerobic spore-forming bacteria. As with the testing of aerobic plate count and lactic acid bacteria, this testing took place over the same storage period.

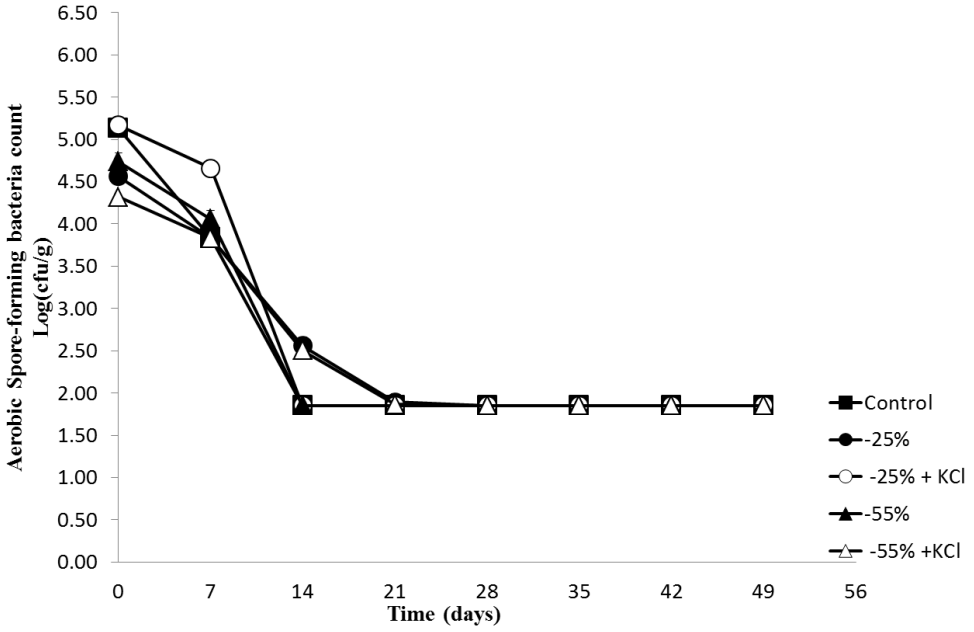

Figure 13: The effect of sodium reduction on the aerobic spore-forming bacteria count of Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 4°C.

Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

The aerobic spore forming bacteria count of Cheddar cheese and low sodium Cheddar cheese stored at 4°C declined from 4.5 log CFU/g to 1.85 log CFU/g (the limit of detection) within 21 days. At 7 days, the spore forming population of cheese made with 25% reduced sodium with KCl replacement was higher than the remaining 4 treatments but by day 14 this difference was not observed (Figure 13). Similarly, at time 14, the aerobic spore forming populations of cheese made with 25% reduced sodium, and 55% reduced sodium with KCl replacement were higher than the remaining 3 treatments, but

by time 21, this difference was not observed. With the exception of these two observations, there was no difference based on salt treatment in aerobic spore forming population in Cheddar cheese and low sodium Cheddar cheese stored at 4°C.

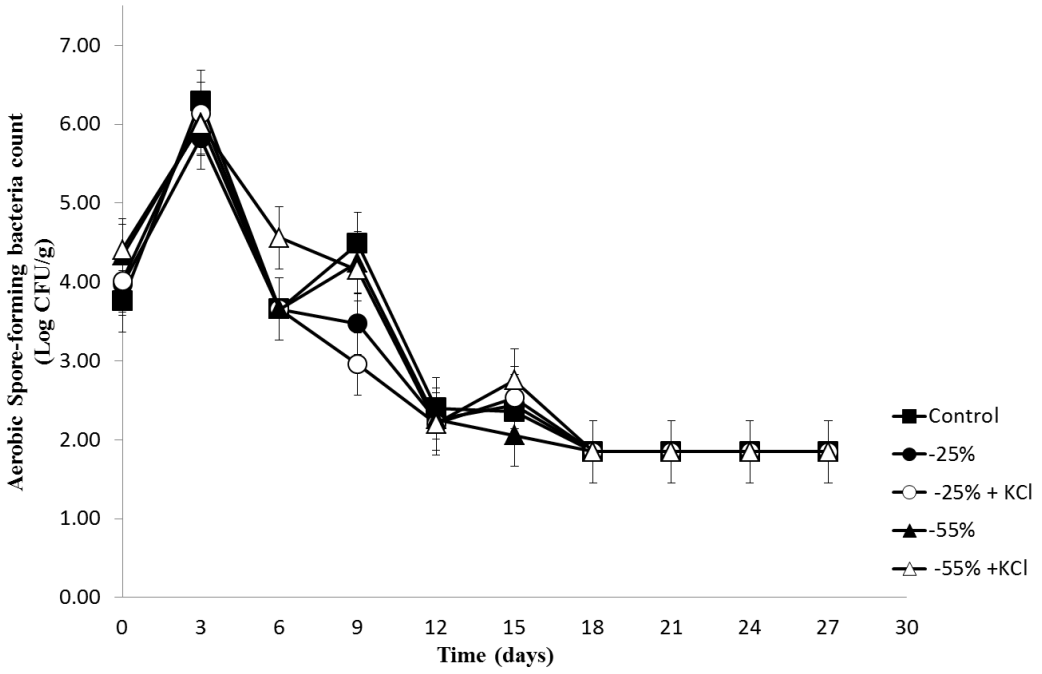

Figure 14: The effect of sodium reduction on the aerobic spore-forming bacteria count of Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 12°C.

Time 0 denotes 153 days after production. Error bars represent standard error; each data point represents the average of 8 plate counts.

In cheese stored at 12°C, the population of aerobic spore forming organisms declined from 4.5 log CFU/g to 1.85 log CFU/g (limit of detection) over the course of 18 days (Figure 14). After 6 days, cheese made with the treatment 55% reduced sodium with KCl replacement had an aerobic spore forming bacteria count higher than that of the

remaining four treatments. With the exception of this observation, although the overall population fluctuated slightly, there was no difference in population of aerobic spore formers based on salt treatment.

3.3.3: *Listeria monocytogenes* in Cheddar Cheese, Reduced and Low Sodium Cheddar Cheese

Listeria monocytogenes was enumerated in the three stages outlined by the experimental design over the course of 9 months. During the first experimental stage, cheese manufactured with the starter cultures *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* was stored at 4°C for 63 days. The experiment began approximately 2 weeks after manufacture.

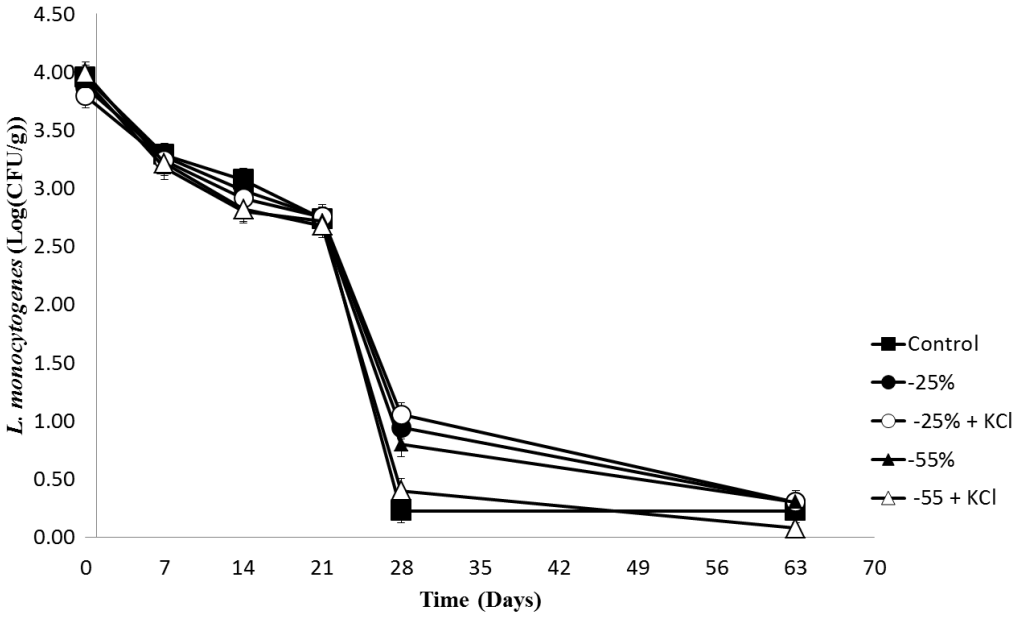

Figure 15: The effect of sodium reduction on the survival of *Listeria monocytogenes* in Cheddar cheese manufactured with *L. lactis* ssp. *lactis* and stored at 4°C during the first 2 months of cheese aging (Stage 1).

Time 0 denotes 13 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

Cheese manufactured with both starter cultures was inoculated at a level of 4 log CFU/g. After inoculation, the *L. monocytogenes* population declined steadily over the entire period of storage (Figures 15 and 16).

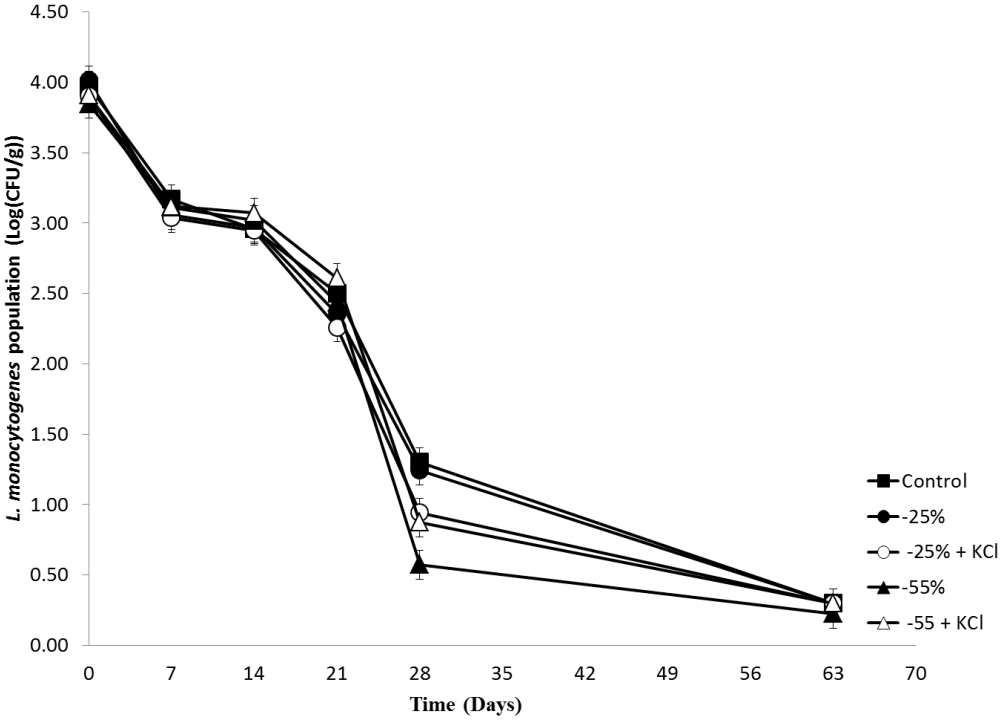

Figure 16: The effect of sodium reduction on the survival of *Listeria monocytogenes* in Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* and stored at 4°C during the first 2 months of cheese aging (Stage 1).

Time 0 denotes 13 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

After 28 days, the population of *L. monocytogenes* fell below the traditional limit of detection. Data shown for days 28 and 63 were obtained using enrichment methods. There was no difference in *L. monocytogenes* survival as a result of salt treatment applied. During the first stage of enumeration, there was no difference in survival observed based on starter culture. As a result, during the second stage, only cheese manufactured using *L. lactis* ssp. *cremoris* was used to study the survival of *L. monocytogenes*. During this experimental stage, cheese was stored at 4° and 12°C.

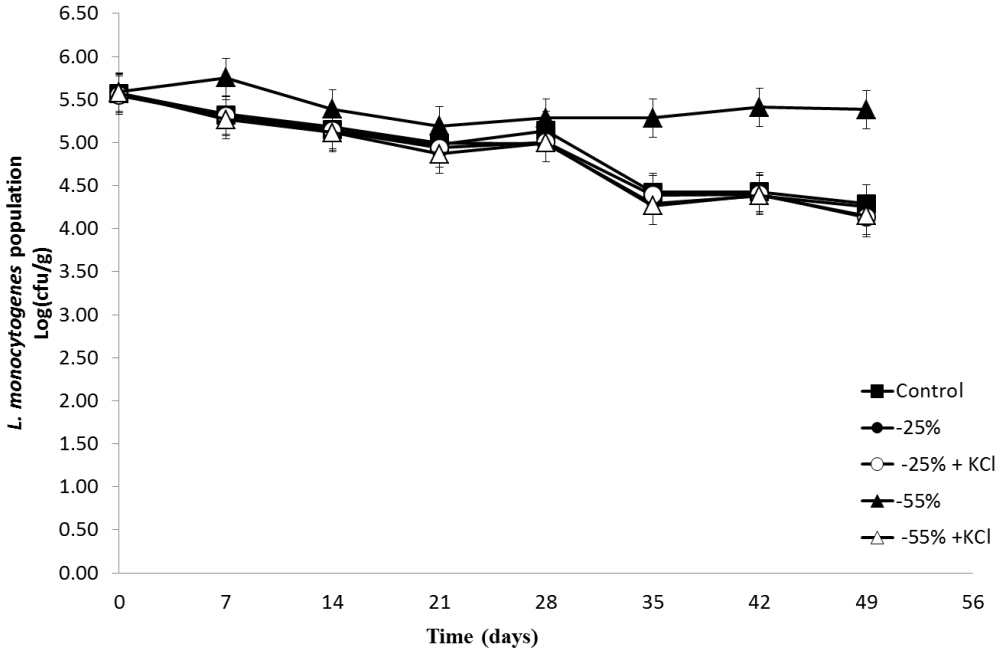

Figure 17: The effect of sodium reduction on the survival of *Listeria monocytogenes* in Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* and stored at 4°C during the 5th and 6th month of cheese aging (Stage 2).

Time 0 denotes 104 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During the second experimental stage, the population of *L. monocytogenes* declined much more gradually than in the first stage. The overall population declined almost 1 log CFU/g from an initial inoculation level of 5.5 log CFU/g, with the exception of the 55% reduced sodium treatment, which supported a consistent population throughout storage (Figure 17). During the first 28 days of testing there was no difference in the survival of *L. monocytogenes* based on salt treatment of the cheese.

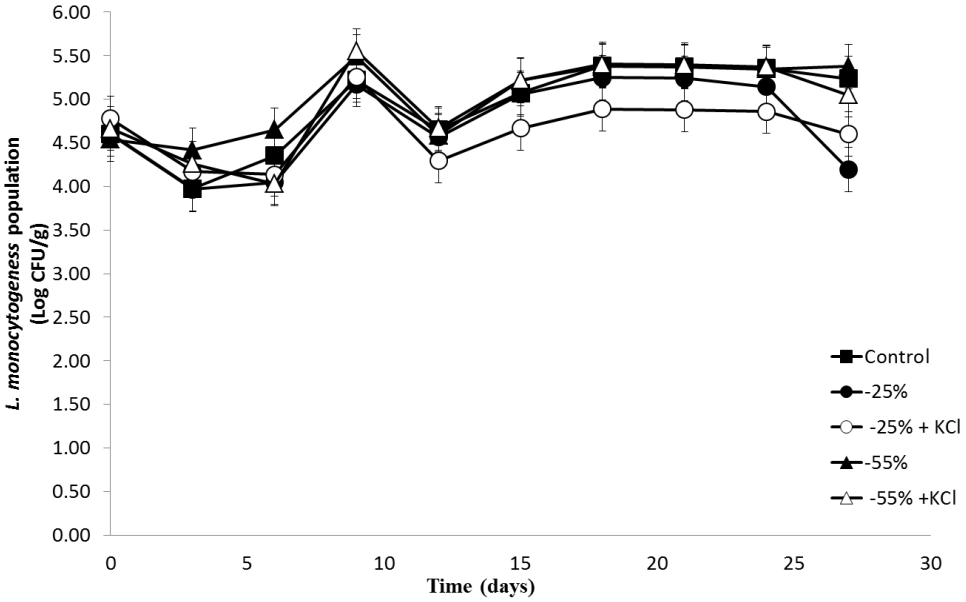

Figure 18: The effect of sodium reduction on the survival of *Listeria monocytogenes* in Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* and stored at 12°C during the 5th month of cheese aging (Stage 2).

Time 0 denotes 104 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

In storage at 12°C, there was no difference in the survival of *L. monocytogenes* based on salt treatment; the population of *L. monocytogenes* was maintained at a level of 5 log CFU/g (Figure 18).

During the final experimental stage, the survival of *L. monocytogenes* was monitored in Cheddar cheese, reduced, and low sodium Cheddar cheese manufactured with both starter cultures during storage at 4°C.

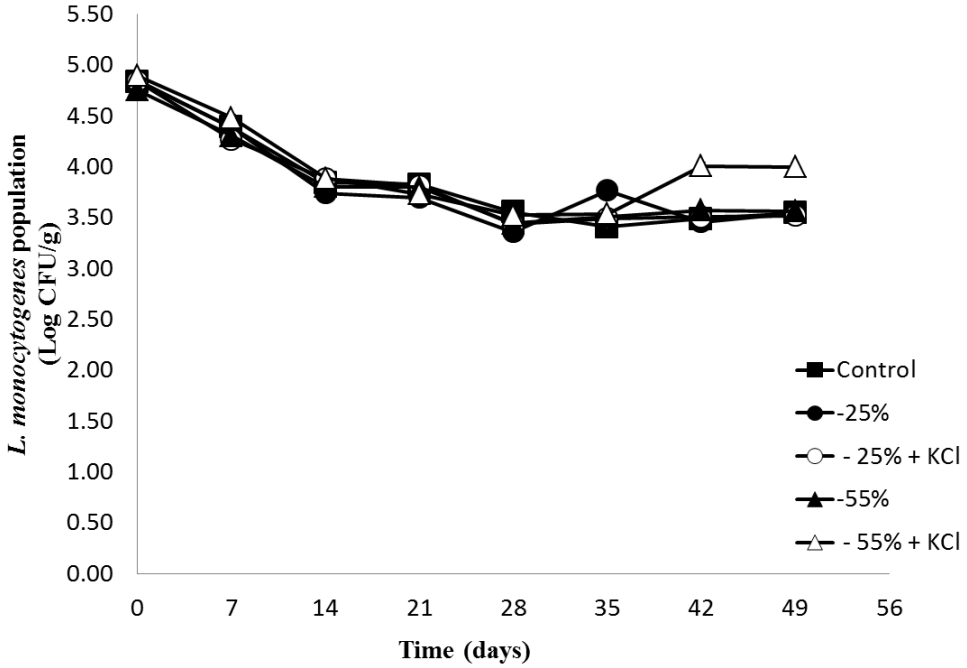

Figure 19: The effect of sodium reduction on the survival of *L. monocytogenes* in Cheddar cheese manufactured with the starter culture *L. lactis* ssp. *lactis* at 4°C during the 7th to 9th month of cheese aging (Stage 3).

Time 0 denotes 243 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During the third experimental stage, the population of *L. monocytogenes* in Cheddar cheese and low sodium Cheddar cheese manufactured using both starter cultures declined gradually from a population of 5 log CFU/g to 3.5 log CFU/g. This trend was observed in all treatments with the exception of cheese manufactured using 55% reduced sodium with KCl replacement. In this treatment, *L. monocytogenes* survived at a level of 4 log CFU/g. During this stage of testing there was no significant difference in survival of *L. monocytogenes* based on starter culture used in manufacture.

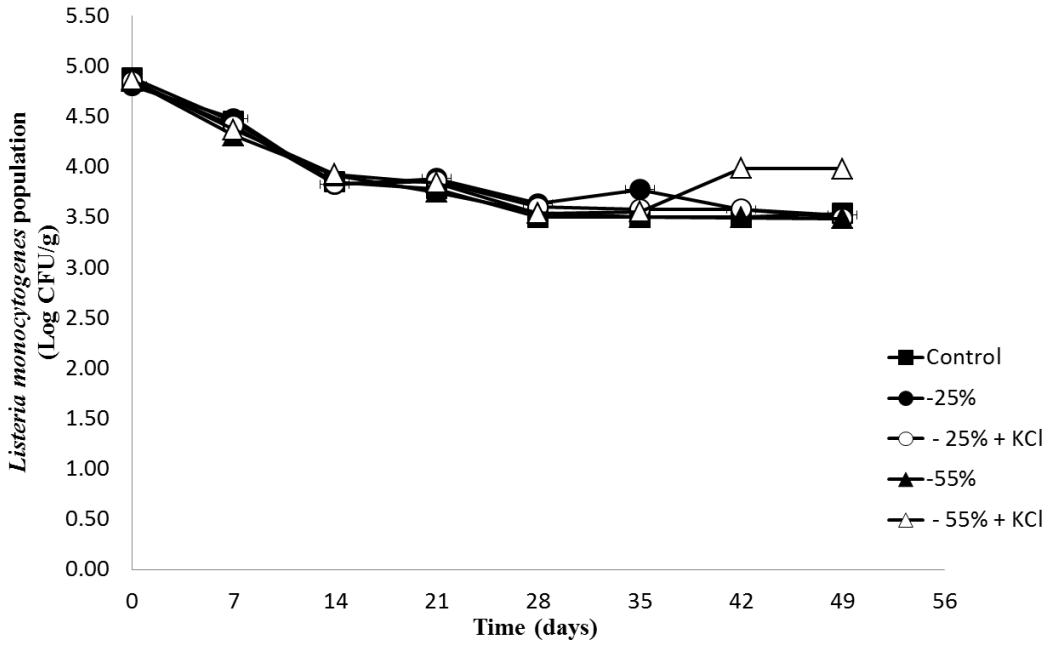

Figure 20: The effect of sodium reduction on the survival of *L. monocytogenes* in Cheddar cheese manufactured with the starter culture *L. lactis* ssp. *cremoris* at 4°C during the 7th to 9th month of cheese aging (Stage 3).

Time 0 denotes 243 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

3.3.4: *Bacillus anthracis* in Cheddar cheese, reduced, and low sodium Cheddar cheese

A series of experiments were performed on Cheddar cheese and low sodium Cheddar cheese inoculated with *B. anthracis* spores. The enumeration of *B. anthracis* also occurred during the three experimental stages. In the first stage of testing, *B. anthracis* spores were used to inoculate Cheddar cheese and low sodium Cheddar cheese manufactured using the starter cultures *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* that

was then stored at 4°C for 63 days.

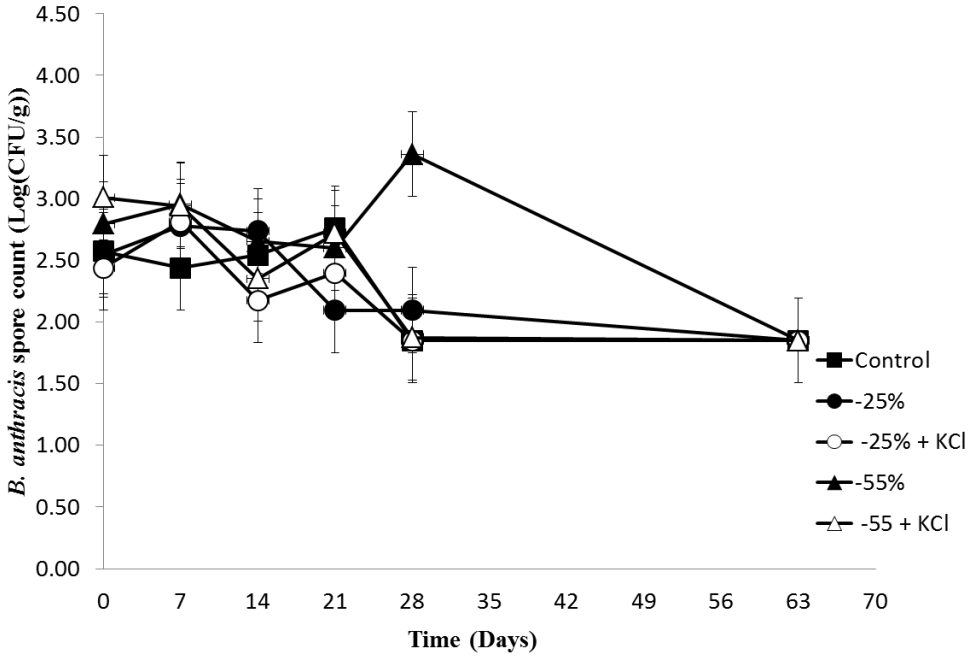

Figure 21: The effect of sodium reduction on the survival of *B. anthracis* spores in Cheddar cheese manufactured using *L. lactis* ssp. *lactis* and stored at 4°C during the first 2 months of aging (Stage 1).

Time 0 denotes 13 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During stage 1, the population of *B. anthracis* declined from 2.5 log CFU/g to 1.85 log CFU/g (limit of detection) (Figures 21 and 22). During this time, there was no difference in the survival of *B. anthracis* in Cheddar cheese based on starter culture or salt treatment with one exception. At day 28 of testing cheese from both starter cultures, the count of *B. anthracis* in cheese treated with a 55% reduction in sodium was higher than the counts obtained for all other treatments.

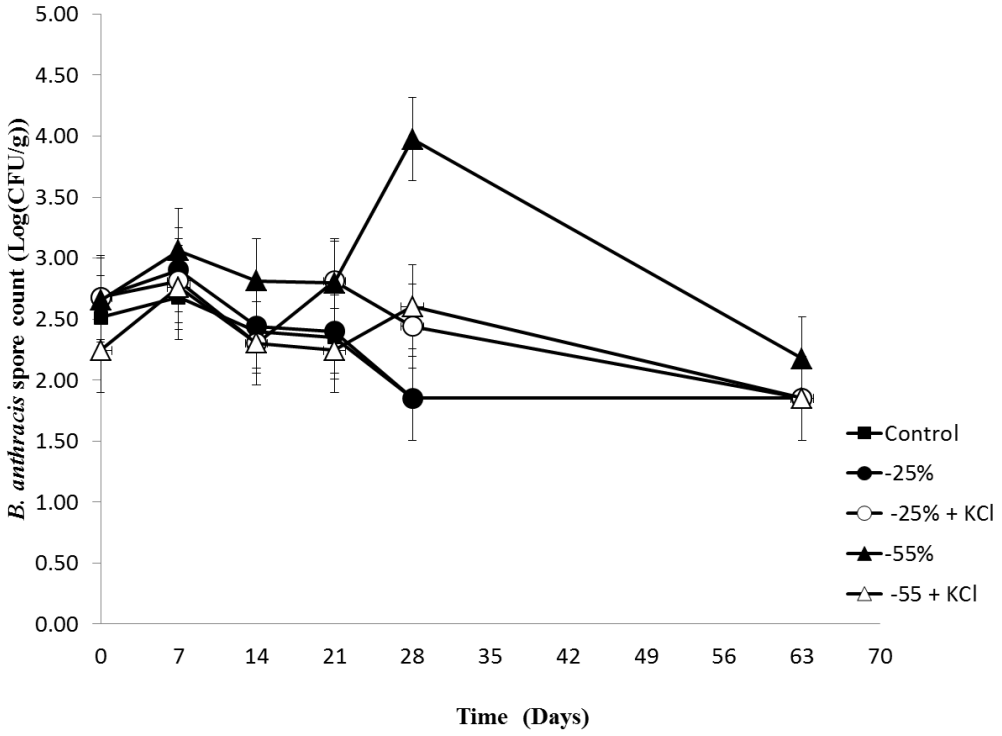

Figure 22: The effect of sodium reduction on the survival of *B. anthracis* spores in Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* stored at 4°C during the first 2 months of aging (Stage 1).

Time 0 denotes 13 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During the second stage of microbiological testing, the survival of *B. anthracis* spores in Cheddar cheese and low sodium Cheddar cheese was monitored only in cheese manufactured using the starter culture *L. lactis* ssp. *cremoris*, as there was no observed difference between survival in cheese made with either starter culture. During stage 2, cheese was stored at 4° and 12°C.

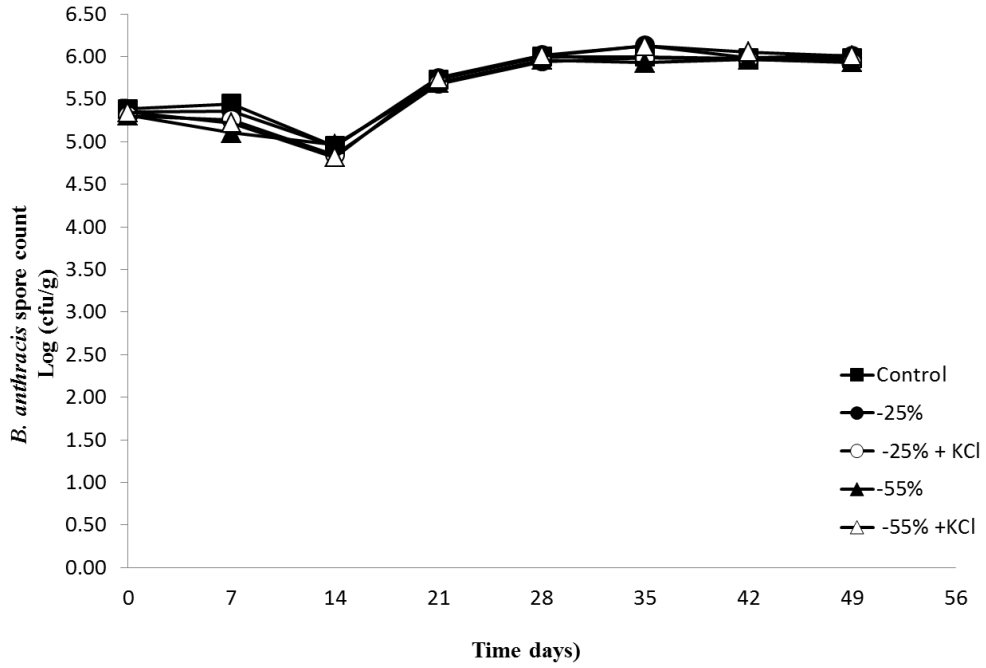

Figure 23: The effect of sodium reduction on the survival of *B. anthracis* spores in Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* stored at 4°C during the 5th and 6th months of aging (Stage 2).

Time 0 denotes 153 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During the second stage of testing, the population of *B. anthracis* in Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* decreased from 5.5 log CFU/g to 5 log CFU/g before gradually increasing to 6 log CFU/g over the course of 49 d (Figure 23). During this time, there was no difference in the survival of *B. anthracis* based on salt treatment. In cheese stored at 12°C, the population of *B. anthracis* decreased only slightly from 6.5 log CFU/g to 6 log CFU/g (Figure 24). At 18 days, the population of *B. anthracis* decreased from 6 log CFU/g to 5.5 log CFU/g before returning to 6 log CFU/g by time

21. During storage at 12°C, there was no difference in the survival of *B. anthracis* based on salt treatment.

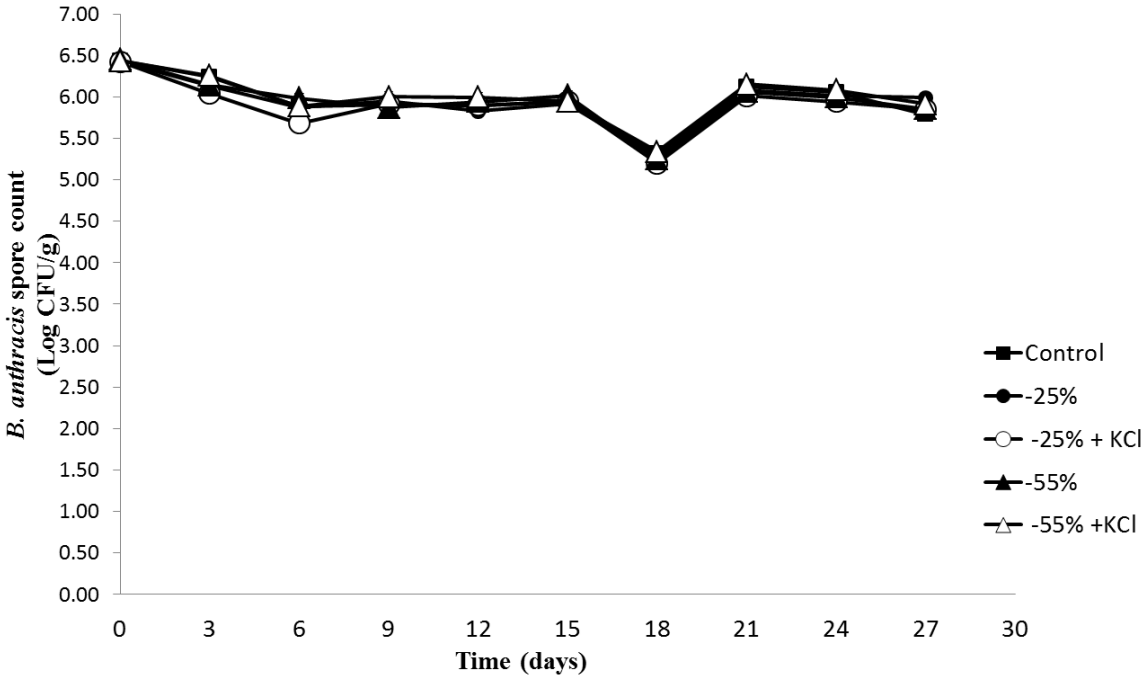

Figure 24: The effect of sodium reduction on the survival of *B. anthracis* spores in Cheddar cheese manufactured with *L. lactis* ssp. *cremoris* stored at 12°C during the 5th month of aging (Stage 2).

Time 0 denotes 153 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

The third stage of microbiological testing was designed to simulate a worst case scenario in which Cheddar cheese that had been contaminated with *B. anthracis* was left at room temperature for over 24 hours. In this experiment, Cheddar cheese, reduced and low sodium Cheddar cheese samples manufactured using the starter culture *L. lactis* ssp.

cremoris were stored at room temperature and the population was enumerated over the course of 32 hours.

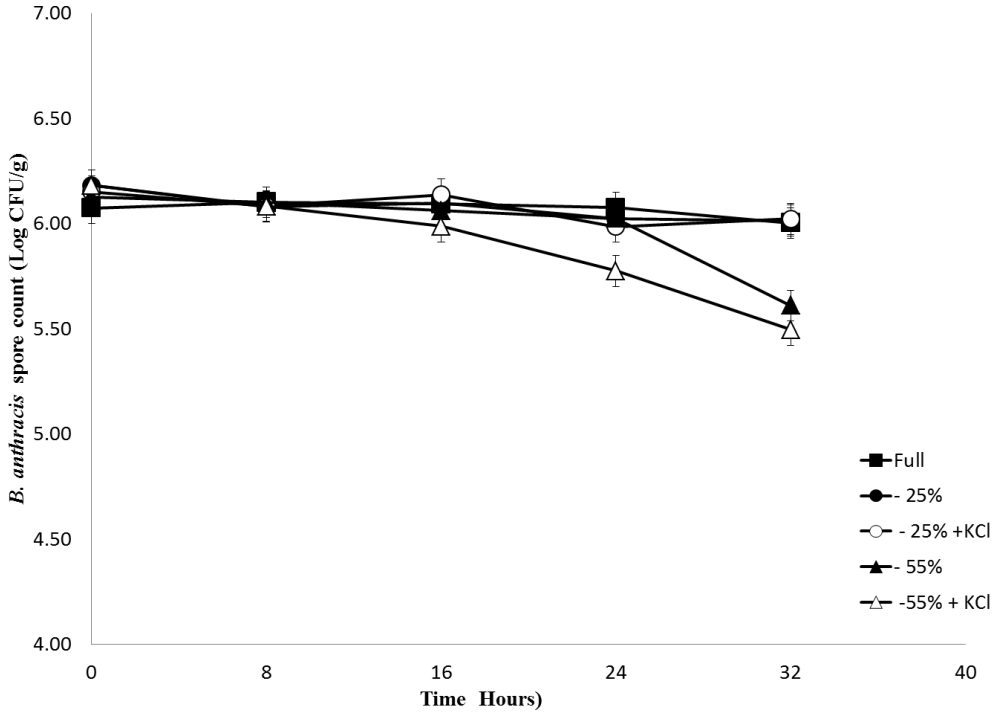

Figure 25: The effect of sodium reduction on the survival of *B. anthracis* spores in Cheddar cheese manufactured using *L. lactis* ssp. *cremoris* and stored at 23°C during the 7th month of aging (Stage 3).

Time 0 denotes 243 days after cheese manufacture. Error bars represent standard error; each data point represents the average of 8 plate counts.

During the third stage of microbiological testing, *B. anthracis* spore counts in Cheddar cheese and low sodium Cheddar cheese were maintained at a level of 6 log CFU/g for 16 h. At 24 h, the count of spores in cheese made with 55% reduced sodium was lower than the counts in the remaining 4 treatments. In the final enumeration (32 hours), the counts

of *B. anthracis* spores in cheeses made with 55% reduced sodium and 55% reduced sodium with KCl replacement were not different from each other, but were lower than those of the remaining three treatments.

3.4: Discussion

The primary goal of this research was to assess the effect of sodium reduction on the microbiological safety of Cheddar cheese. The safety of a food product is affected by many elements of food processing and distribution. The parameters investigated here were the survival of *Listeria monocytogenes* and *Bacillus anthracis* spores in Cheddar cheese as influenced by reduction of sodium chloride. To accomplish this goal, stirred curd Cheddar cheese was manufactured with two sodium reduction levels—with and without potassium chloride replacement. Sodium reduction levels of 25 percent and 55 percent were chosen in accordance with the respective "reduced sodium" and "low sodium" claims that could be made (FDA, 2009; Johnson et al., 2009). After manufacture, cheese samples were inoculated with mixtures of *L. monocytogenes* strains and *B. anthracis* spores. The viable cell and spore counts of these pathogens were monitored over time respectively. In addition to the monitoring of pathogenic organisms in cheese, uninoculated samples were analyzed for various physical, chemical and microbiological characteristics, as these may play a role in the growth or survival of pathogens in contaminated food products.

3.4.1: Chemical composition of Cheddar cheese, reduced, and low sodium Cheddar cheese

The chemical composition of Cheddar cheese may be affected by a reduction in sodium. No difference in the fat or protein content of the cheese was observed based on starter culture used in manufacture or salt treatment applied (Table 5). The moisture content in the cheese did not vary based on salt treatment until after 85 days of storage. After this period of storage, the moisture decreased from 37.5% to 34.5%, and varied only slightly in cheese manufactured using *L. lactis* ssp. *lactis* (Figures 4 and 5). Typically, sodium reduction in Cheddar cheese leads to increased moisture because reduction in sodium leads to reduced syneresis and increased moisture retention (Fox et al., 2000).

In Cheddar cheeses, Thakur et al. (1975) found the moisture values of unsalted Cheddar cheese were higher than the moisture values of salted Cheddar cheese after 12 weeks of aging. This result was corroborated by the research performed by Schroeder et al. (1988). In a study of Cheddar cheese made with 5 different salting treatments (ranging from unsalted to 1.44% NaCl), Schroeder et al. (1988) found that decreasing the amount of NaCl added to the cheese directly correlated with an increase in the initial moisture. Similarly, Shrestha et al. (2011) found that a reduction in the amount of sodium used during manufacture resulted in an increase in moisture.

It is likely that no difference in moisture was observed due to manufacturing differences between Cheddar cheese and stirred curd Cheddar cheese. In the manufacture of stirred curd Cheddar cheese (as in this study) there is no Cheddaring step and the curds

are kept to a small size, which exposes a greater curd surface area, and presumably allows for rapid penetration of salt into the curd particle, resulting in the same amount of moisture loss at salting. As a result, curds are better able to expel moisture than those that have been Cheddared and milled into larger pieces (having a smaller surface to volume ratio) (ur-Rehman et al., 2008). In a study of Cheddar cheese made with reduced sodium and KCl substitution, Reddy and Marth (1995) observed no difference in moisture between samples based on salt treatment (with the exception of cheese treated with no sodium) .

No difference in the initial pH was observed based on salt treatment. In Cheddar cheese made with varying levels of NaCl and KCl replacement, Reddy and Marth (1995) also observed no difference in pH based on salt treatment. In Cheddar cheese made with sodium reduction and KCl replacement based on ionic strength, after 16 weeks of aging, Fitzgerald and Buckley (1985) found the pH of full sodium cheese to be no different than the pH of cheese made with KCl alone, or a 1:1 mixture of NaCl and KCl. Over ten months of storage, the pH of all samples increased gradually from an average of 4.65 to an average of 5.30 (Figure 8). It is likely that the observed increase in pH was due to proteolysis. After a short period of aging starter cultures are inhibited by the acidic conditions of the cheese and are no longer present in significant numbers. As the starter culture organisms begin to die, non-starter lactic acid bacteria (NSLAB) increase in population. During proteolysis, these organisms produce amino acid decarboxylases and deaminases and ultimately produce alkaline compounds, causing the pH to increase (Kosikowski and Mistry, 1999).

The initial water activity of the cheese was measured, but this value was omitted due to technical issues. Water activity was measured again after 1, 2, and 3 months of storage at 4°C. During this time, the water activity stabilized near 0.94. The reduction of sodium from stirred curd Cheddar cheese and replacement by KCl did not result in a significant difference in water activity. Previously, substitution of NaCl with KCl in cheese has been reported to cause the water activity to increase (Cruz et al., 2011). Although not observed in this study, it is likely that an observed increase in water activity could be due to the difference in molecular weights of Na and K. As previously discussed, sodium has a lower molecular weight than K, and at an equivalent mass is better able to lower the water activity of a system because there are more molecules of NaCl than KCl (Grummer and Schoenfuss, 2011). Ultimately, the levels of Na reduction and K replacement in this study were chosen in an effort to maintain the water activity of the cheese at a level near that of the full sodium cheese. Sodium reduction and potassium chloride replacement did not significantly affect the composition of stirred curd Cheddar cheese over the course of 85 days of storage.

3.4.2: Analysis of natural microbiological indicators and background flora

The aerobic plate count (APC) of cheese stored at 4°C varied slightly by treatment during initial storage, but after 28 days, the population was more uniform among treatments, at a level of 5 log CFU/g (Figure 9). The APC in cheese stored at 12°C was uniform for all salt treatments throughout the entire storage period, at a level of 5.5 log CFU/g (Figure 10). Reddy and Marth (1995) also observed no difference in APC in Cheddar cheese made with varying levels of sodium reduction and KCl replacement

based on salt treatment during 36 weeks of aging at any one ripening period. Although a slight decline was observed, in a study of Cheddar cheese during six months of aging, Haque et al. (1997) did not observe a significant change in APC of samples. The counts obtained by Haque et al. (1997) were similar to those obtained in this study. The level of population and survival observed in this study is similar to that found in by Aly et al. in Feta cheese (1995), and by de Lima et al. (2011) in grated cheese made and sold in Brazil.

The population of lactic acid bacteria in all cheese treatments stored at both 4° and 12°C was between 8 and 8.5 log CFU/g throughout storage (Figures 11 and 12). This result is consistent with similar studies of lactic acid bacteria in stirred curd Cheddar cheese and conventional Cheddar cheese (Turner and Thomas, 1980; Schroeder et al., 1988; Hou et al., 2012). There was no noticeable fluctuation in population of lactic acid bacteria during the storage of cheese at 4° or 12°C. This was likely due to the population dynamics of lactic acid bacteria. Initially after manufacture, the population of lactic acid bacteria comprised of the starter culture was approximately 7 to 8 log CFU/g. In general, during the first two weeks of Cheddar cheese aging, this population is believed to decline rapidly, as the population of NLSAB increases rapidly, even surpassing this level to populations of 8 or 9 log CFU/g (Fox et al., 2000). Because the decline in starter culture lactic acid bacteria occurs at the same time as the increase in NSLAB, the total lactic acid bacteria population appears to remain constant.

The population of aerobic spore-forming organisms in Cheddar cheese stored at 4° and 12°C declined steadily during the first three weeks of storage (Figures 13 and 14)

from an initial count of almost 6 log CFU/g to undetectable levels after less than a month. This result is inconsistent with data obtained by Reddy and Marth (1995) from a similar study of sodium reduction and KCl replacement in Cheddar cheese. According to that study, the initial measurement of spore-forming organisms was 3.75 log CFU/g. After two weeks of ripening, the population declined slightly to 3 log CFU/g and remained at 3.75 log CFU/g for the duration of testing. It is possible that the stress of the preparation of cheese for further microbiological testing (physical manipulation by shredding, temperature changes during preparation) caused the spores to germinate. Upon germination, the conditions of the cheese may have been too harsh to permit outgrowth, and did not require sporulation, resulting in the observed decline in spore population.

Modifications to the sodium levels did not significantly affect the natural microbial flora monitored in Cheddar cheese (APC, lactic acid bacteria, and aerobic spore-forming bacteria). Several of these organisms, especially lactic acid bacteria, are important to the formation of desirable flavor compounds in the cheese during ripening.

3.4.3 *Listeria monocytogenes* in Cheddar cheese, reduced, and low sodium Cheddar cheese

The population of inoculated *L. monocytogenes* in Cheddar cheese and reduced sodium Cheddar cheese was monitored in three stages (Figure 3). During experimental stage 1, cheese manufactured with both starter cultures was inoculated with *L. monocytogenes* and stored at 4°C for 63 days. During this time, the population of *L. monocytogenes* declined from an initial level of inoculation of 4 log CFU/g to less than 1 log CFU/g (Figures 15 and 16). While the value of 1 log CFU/g is an estimate based on

the enrichment procedures used (Schamberger et al., 2004), it is still very clear that the population declined from the beginning to the end of the experiment was significant. Because the pH of cheese was lowest during stage 1, we can speculate that the cause of the marked decline in the population of *L. monocytogenes* was the sensitivity of this bacterium to acidic conditions. During this period of testing, the pH of the cheese only increased slightly from 4.75 to 4.90 (Figure 8).

It has been long recognized that some strains of lactococcal cheese starter cultures are able to produce bacteriocins. Certain strains of the genus and species *Lactococcus lactis* are able to produce nisin (Bellow et al., 2012). Nisin has been shown to inhibit the survival of Gram positive foodborne pathogens such as *L. monocytogenes* (Gadotti, 2011). While it is possible that bacteriocins produced by the starter cultures may have contributed to the decline in *L. monocytogenes* counts, these starter cultures were not analyzed for their ability to produce nisin. Furthermore, by the time of inoculation, the cheese was already 13 days old. While the lifespan of lactococcal starter cultures in Cheddar cheese is not known, several studies have shown it to be between 1 and 30 days (McSweeney et al., 1993; Reddy and Marth, 1995; Haque et al, 1997). The sharpest decline in *L. monocytogenes* population took place after the starter culture activity was likely to have greatly declined. While bacteriocins may have been produced by the starter culture organisms, it is likely that the low pH of the cheese during this phase of testing most strongly influenced the decline in *L. monocytogenes* population.

While similar reductions of L. monocytogenes have been reported before, it appears that the time of inoculation has significant impact on *L. monocytogenes* counts.

In a study by Ryser and Marth (1987a) using a relatively low inoculum of 100 CFU/g at the moment of Cheddar manufacture, the population increased to 1,000 CFU/g after 14 days but then declined to levels that could only be recovered by enrichment. However, viable counts were still detected for more than 100 days. In a more recent study, during 90 days of storage at 4°C Shrestha et al. (2011) observed a significant decline in the population of *L. monocytogenes* added to Cheddar cheese as a post-manufacturing contaminant. Chihuahua cheese is made in Mexico, and is similar to Cheddar cheese in that both types of cheese undergo a cheddaring step. Mexican Manchego cheese is more similar to stirred curd Cheddar cheese, as the cheddaring step is absent. In a study of the fate of *L. monocytogenes* introduced during the manufacturing of these cheeses, it was determined that *L. monocytogenes* was able to survive well during 5 days of ripening in Mexican Manchego cheese, and 6 weeks of ripening in Chihuahua cheese (Solano-Lopez and Hernandez-Sanchez, 2000). The population of *L. monocytogenes* declined only 1 log CFU/g from the start of manufacture to the end of ripening in Mexican Manchego cheese, and 2 log CFU/g from the start of manufacture in Chihuahua cheese. It can be speculated then, that at similar population levels, *L. monocytogenes* would behave in a similar manner in stirred curd and traditional Cheddar cheese.

After 28 days of storage, there were statistically significant differences in population based on salt treatment (Figures 15 and 16). It is possible that the use of the enrichment procedures noted above was the cause of these differences, and it is unlikely that these differences are truly based on the salt treatment applied. This theory is confirmed by the aforementioned study by Shrestha et al. (2011). While a significant

decline in population was observed, the authors noted that there was no significant difference in *L. monocytogenes* population due to sodium reduction.

No significant difference in *L. monocytogenes* population due to starter culture was observed during stage 1, so in the second stage of this project, only cheese manufactured using the starter culture *L. lactis* ssp. *cremoris* was inoculated with *L. monocytogenes.* In storage at 4°C, the population of *L. monocytogenes* only declined from 5.5 log CFU/g to 4 log CFU/g in all treatments except for cheese made with a 55% reduction in sodium (Figure 17). Cheese treated with a 55% reduction in sodium allowed for the survival of *L. monocytogenes* at a population level of 5.25 log CFU/g. It is likely that an increase in pH from 4.7 in phase 1 to 5.25 in phase 2, in combination with reduced sodium allowed for the increased survival of *L. monocytogenes* in cheese made with 55% reduced sodium. The increased pH during this phase of testing also permitted the increased survival of *L. monocytogenes* in the remaining four treatments. The optimum pH for *Listeria monocytogenes* is 7.0 (Fox et al., 2000). As the pH of the cheese became less acidic and closer to the optimum pH of *L. monocytogenes*, the bacterium was better able to survive in the cheese. This theory is supported by results obtained by Ryser and Marth (1987b) in their study of *L. monocytogenes* in Camembert cheese. Camembert cheese is known to have a pH higher than that of Cheddar due to the secondary mold ripening. In their study, Ryser and Marth showed survival and growth of *L. monocytogenes* in Camembert cheese stored at 6°C for 65 days. Moreover, the a_w of the cheese at this phase of testing plateaued near 0.94, a level conducive to the survival of *L. monocytogenes*.

In a more recent study of Camembert cheese, D'Amico et al. (2008) found that when introduced as a post-processing contaminant, *L. monocytogenes* was able to survive in Camembert cheeses made with raw and pasteurized milk. The bacterium was able to survive longer than 60 days in both types of cheese, and at a low and high inoculation level. The current federal requirement for aging of raw milk is that the cheese must be held for 60 days at approximately 1.7°C (35°F) (Code of Federal Regulations, 2006). The results from D'Amico et al. (2008) indicates that cheeses like Cheddar and Camembert may need to be aged significantly longer than 60 days when manufactured with raw milk.

During stage 2, when stirred curd Cheddar cheese was stored at 12°C, no significant increase or decrease in population was observed for almost a month of storage. Additionally, there was no difference in *L. monocytogenes* population based on salt treatment (Figure 18). As previously discussed regarding cheese stored at 4°C, it is likely that the increased pH of the cheese permitted this survival. In addition to their study of Camembert cheese, Ryser and Marth (1989) also studied the fate of *L. monocytogenes* in Brick cheese. When stored at 15°C for 28 days, it was determined that milk inoculated with 2 or 3 log CFU/ml *L. monocytogenes* after pasteurization could result in *L. monocytogenes* populations greater than 6 log CFU/g in brick cheese, and then survival at similar levels for over 12 weeks (Ryser and Marth, 1989).

In the final experimental stage, *L. monocytogenes* was used to inoculate cheese manufactured using each of the starter cultures. These samples were then placed in 4°C storage for 49 days. During this storage period, the population of *L. monocytogenes* declined from an initial inoculation level of 5 log CFU/g to 3.5 log CFU/g (Figures 19

and 20). It should be noted that the difference in survival between stages 2 and 3 appeared to be related to changes in pH. The cheese pH declined from maximum values of approximately 5.3 to 5.4 at an age of 180 days during stage 2 to values of 5.0 to 5.25 by day 225 right before the start of stage 3. During stage 2 only a slight reduction was observed in some of the treatments, but in stage 3 a 1.5 log CFU/g reduction was consistently observed.

The starter culture used in manufacture did not significantly affect the population of *L. monocytogenes* at any time point. There was no difference in *L. monocytogenes* population based on salt treatment until day 35, at which point the population of *L. monocytogenes* in cheese manufactured with a 25% reduction in sodium was slightly higher than that of the remaining four treatments. Although this difference was significant, it was less than 0.5 log CFU/g higher, and the difference was not observed at any other time point.

After 42 days of storage, the population of *L. monocytogenes* in cheese made with 55% reduced sodium with KCl replacement was significantly greater than that of the remaining four treatments. While a slight decline in *L. monocytogenes* was observed, the average population seemed to plateau and survive at 3.5 log CFU/g after 28 days of storage at 4°C. This survival can be attributed to the relatively high pH of the cheese at this time period (Figure 8) as discussed regarding the results of stage 2 of *L. monocytogenes* testing. These results are consistent with those found by Shrestha et al. (2011). When *L. monocytogenes* was used as a post-processing contaminant of Cheddar cheese stored at 4°C, the survival of the pathogen was greater in the low salt, high pH

treatment than in remaining treatments (low salt, low pH; standard salt, high pH; standard salt, low pH), but *L. monocytogenes* was able to survive at or near the initial level of inoculation.

The survival of *L. monocytogenes* was not largely affected by sodium reduction or KCl replacement. Increased survival was correlated with an increase in the pH of the cheese due to natural ripening processes.

3.4.4 *Bacillus anthracis* in Cheddar cheese, reduced, and low sodium Cheddar cheese

It has been established that the spores of *B. anthracis* are able to survive pasteurization (Xu et al., 2006; Leishman et al., 2010b). It is possible that milk could be contaminated naturally, due to presence of *B. anthracis* in soil used for dairies (Erickson and Kornacki, 2003). Thus, if milk were intentionally or unintentionally contaminated with *B. anthracis*, before or after pasteurization, and if that milk were used to manufacture Cheddar cheese, the cheese would also become contaminated with *B. anthracis.* To determine the outcome of such a hypothetical scenario, the fate of *B. anthracis* spores in Cheddar cheese and low sodium Cheddar cheese was studied in two stages with a short final experiment to conclude the research (Figure 3).

During the first experimental stage, stirred curd Cheddar cheese samples, manufactured using the two different starter cultures, were inoculated with *B. anthracis* spores and placed in 4°C storage for 63 days. Over the course of this storage period, the *B. anthracis* spore count slowly declined from an average initial inoculation level of 3 log CFU/g to 1.85 log CFU/g. This decline was not a result of the starter culture used in

manufacture or the salt treatment applied to the cheese. In a study of *B. anthracis* in fruit juice and wine, *B. anthracis* spores survived within 1 log CFU/g of the initial inoculation level for 30 days when stored at 4°C (Leishman et al., 2010a). The pH of these liquid foods was between 2.8 and 4.3; *B. anthracis* is an organism readily able to survive harsh conditions, including the relatively low pH of cheese.

After 28 days of storage, the *B. anthracis* spore count in cheese made with a 55% reduction in salt was significantly higher than that found in the remaining four treatments. Sodium reduction likely permitted a slightly increased level of survival. However, it is difficult to fully attribute this result to sodium reduction, as the difference in survival was not observed at the next sampling, or any other sampling occasions in this experimental stage. This finding is similar to that of Khalil et al. (1993): in a study of the response of *B. cereus* to varying concentrations of salt and other sodium and potassium based curing agents in nutrient broth, the authors found that increased salt did not significantly affect the survival of *B. cereus* spores. Because of the great similarity between *B. anthracis* and *B. cereus*, it is likely that *B. anthracis* would respond in a similar matter.

The level of inoculation of *B. anthracis* in stage 1 of microbiological testing did not allow for substantial observation of the survival or decline of the spore counts for *B. anthracis* in cheese, as the limit of detection was reached quickly in all treatments. In experimental stage 2, the initial level of inoculation was increased slightly, and cheese manufactured using *L. lactis* ssp. *cremoris* was inoculated with *B. anthracis* spores and stored at 4° and 12°C for 49 and 27 days respectively. During this storage period, there was no difference in population at any sampling date due to salt treatment (Figures 23

and 24). There were some fluctuations in population during this storage period, but the population of *B. anthracis* spores never decreased by more than 1 log CFU/g from the initial level of inoculation.

This observation of a consistent population of *B. anthracis* throughout storage has been observed in similar studies when products are maintained at low temperature. In a study of *B. cereus* spores in pasteurized milk stored between 5° and 9°C, the population of spores decreased only slightly from 7 log CFU/g to 6 log CFU/g in 48 hours. In raw milk, the *B. cereus* spores were observed to maintain a constant population near 6 log CFU/g when the milk was stored at refrigerated temperatures for 48 hours (Bowen and Turnbull, 1992). These findings support the potential for *B. anthracis* spores to survive in cheese, as they readily survive in a dairy matrix. Additionally, it has been confirmed that *B. anthracis* vegetative cells are destroyed during pasteurization, but spores are not (Perdue et al., 2003; Xu et al., 2006). Perdue et al. also found that even when milk was submitted to pasteurization conditions twice within 24 hours, there was still no effect on the *B. anthracis* spore counts.

During the final phase of microbiological testing, the goal was to simulate a "worst-case scenario" in which Cheddar cheese contaminated with *B. anthracis* spores was left out at room temperature (23°C) for 32 hours and not discarded. During this time, the spore count of *B. anthracis* in cheese manufactured using *L. lactis* ssp. *cremoris* remained constant at the initial level of inoculation (6 log CFU/g). The effect of storage at room temperature did not appear to enhance the growth or survival of *B. anthracis* spores when cheese was stored at 23°C. However, the duration of storage was not long

enough to observe the true effect of increased temperature storage. In a study of sterile, raw ground beef stored at varying temperatures, Tamplin et al. (2008) found that storage of *B. anthracis* contaminated beef at temperatures below 16°C caused a slow decline in spore count, while storage between 17°C and 44°C allowed for a slow rate of growth. If Cheddar cheese were consistently left at elevated temperatures, it is likely that *B. anthracis* spore counts would slowly increase.

No difference in the spore counts was observed as influenced by salt treatment until the samples had been at room temperature for 24 hours (Figure 25). At that time, the count of *B. anthracis* spores in cheese made using a 55% reduction in sodium was lower than that of the remaining four treatments. After 32 hours, the number of *B. anthracis* spores in cheese made using a 55% reduction in sodium, as well as a 55% reduction in sodium with KCl replacement, were not different from each other, but were significantly lower than the remaining three treatments. While this difference was found to be significant, the actual decline in population was less than or equal to 0.5 log CFU/g. It is possible that the reduction in sodium caused this decline in spore count. Additionally, data from Khalil et al. (1993) suggest that a small amount of sodium chloride (2-4%) actually promotes the survival of *B. cereus* spores. Salt is usually applied to Cheddar cheese at a level of 2% during manufacture. Thus a 55% reduction in salt could have negatively affected the survival of *B. anthracis* during this experiment. Ultimately, the difference in spore counts between the treatments was so small that it is also possible that the difference was not fully attributable to sodium reduction.

In summary, *Bacillus anthracis* spores were able to survive in stirred curd Cheddar cheese of varying salt treatments for as long as 49 days when introduced as a post processing contaminant. This survival was not affected by the starter culture used to manufacture the cheese, or the storage temperature of the cheese.

Chapter 4: Concluding remarks

Cheddar cheese is widely consumed in the United States. It is thought to be a very safe product, due to a relatively high sodium content of 600 mg/100g. The consumption of sodium among Americans has become a cause for concern among public health thought leaders who now recommend a reduction in sodium from the American diet.

Listeria monocytogenes is a ubiquitous pathogenic organism that is often found in dairy processing facilities. It may easily contaminate products like Cheddar cheese if the cheese is made from raw milk, or via post-processing contamination if cheese milk is pasteurized. *Bacillus anthracis* is a class A bioterrorism agent, and the etiologic agent of anthrax. The spores of *B. anthracis* are able to survive pasteurization, and the spores of this organism could be used to contaminate the milk supply and ultimately contaminate a product like Cheddar cheese.

To determine the effect of sodium reduction on the safety of Cheddar cheese, full sodium, reduced sodium, and low sodium stirred curd Cheddar cheese was manufactured, and separately inoculated with *L. monocytogenes* and *B. anthracis.* Prior to inoculation, compositional data was collected. Initial measurements of fat, protein, and moisture were not different based on salt treatment. This is encouraging to cheese manufacturers, as it suggests that the composition of Cheddar cheese may not be grossly affected by sodium reduction and replacement by KCl.

Once inoculated, the survival of these organisms in cheese placed in refrigerated storage (temperatures of 4° and 12°C) was monitored in three phases over the course of

27 to 63 days. During the initial phase of testing, the *L. monocytogenes* population declined by nearly 4 log CFU/g, while the population of *B. anthracis* declined slowly by 1.25 log CFU/g. These differences were not attributed to salt treatment, but rather the pH of the cheese (near 4.7).

In the second and third phases of testing, the pH of the cheese had risen to 5.1 and continued to rise throughout testing to 5.3. During these phases, the populations of *L. monocytogenes* and *B. anthracis* were able to survive much better. When stored at 4° and 12°C, the population of *L. monocytogenes* declined by only 1.5 to 2.5 log CFU/g (depending on phase), with few differences in population based on salt treatment. Under these same conditions, the population of *B. anthracis* declined by only 0.5 to 1 log CFU/g (depending on the phase), with few differences in population based on salt treatment.

In addition to monitoring the survival of these two pathogenic organisms, the natural flora present in the cheese was also monitored. This was performed via the measurements of total aerobic count, lactic acid bacteria count, and natural spore forming organisms count. Lactic acid bacteria function as important organisms during the formation of flavor of the cheese during ripening. The survival of these and other background flora was not affected by sodium reduction. Thus, sodium reduction may affect the flavor of the cheese, but it is not because of presence or absence of desirable microorganisms.

These data suggest that the low or standard salt levels used in this study did not greatly affect the survival of *L. monocytogenes* or *B. anthracis* at the experimental incubation temperatures. That is, sodium reduction may not compromise the safety of

food products like Cheddar cheese. These results present an exciting opportunity for the food industry to move forward in meeting sodium reduction initiatives. This progression must take place with caution. Although sodium reduction did not affect the survival of the organisms, under some experimental conditions, *L. monocytogenes* and *B. anthracis* were able to survive in the cheese. This points to the great need for strict adherence to good manufacturing practices and careful handling of products like Cheddar cheese in order to prevent them from becoming contaminated in post processing operations such as slicing or shredding.

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Appendix

A.1: Chemical analysis of full, reduced, and low sodium Cheddar cheese with and without potassium chloride replacement

A.1.1: Moisture

Table 6: Moisture results (Data for table 5, figures 4, 5)

			Days			
Salt System	Starter Culture	Replicate	0	24	50	85
Full Sodium	*L. lactis* ssp. *lactis*	1	39.12	39.43	34.55	33.74
Full Sodium	*L. lactis* ssp. *lactis*	2	37.78	38.00	34.05	33.14
-25%	*L. lactis* ssp. *lactis*	1	37.04	39.28	32.44	30.89
-25%	*L. lactis* ssp. *lactis*	2	37.36	36.73	32.95	28.93
- 25% + KCl	*L. lactis* ssp. *lactis*	1	37.25	37.22	32.88	31.55
- 25% + KCl	*L. lactis* ssp. *lactis*	2	36.98	38.12	32.82	31.18
-55%	*L. lactis* ssp. *lactis*	1	36.19	36.74	31.84	30.44
-55%	*L. lactis* ssp. *lactis*	2	37.83	37.99	33.61	28.37
- 55% + KCl	*L. lactis* ssp. *lactis*	1	36.21	35.68	32.88	28.45
- 55% + KCl	*L. lactis* ssp. *lactis*	2	36.59	36.68	32.73	28.23
Full Sodium	*L. lactis* ssp. *cremoris*	1	38.64	39.63	35.68	29.72
Full Sodium	*L. lactis* ssp. *cremoris*	2	39.20	39.06	34.38	33.88
-25%	*L. lactis* ssp. *cremoris*	1	38.81	39.12	35.25	31.80
-25%	*L. lactis* ssp. *cremoris*	2	39.34	39.60	35.44	32.68
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	38.05	38.58	35.09	32.49
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	39.10	39.66	36.10	34.00
-55%	*L. lactis* ssp. *cremoris*	1	39.99	40.30	36.83	32.93
-55%	*L. lactis* ssp. *cremoris*	2	38.82	38.88	34.93	28.93
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	36.87	37.74	32.75	31.74
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	40.04	40.43	36.14	32.01

A.1.2: Water activity

Table 7: Water activity (figures 6, 7)

			Days		
Salt System	Starter Culture	Replicate	37	62	98
Full Sodium	*L. lactis* ssp. *lactis*	1	0.92	0.91	0.92
Full Sodium	*L. lactis* ssp. *lactis*	2	0.94	0.93	0.93
-25%	*L. lactis* ssp. *lactis*	1	0.92	0.94	0.94
-25%	*L. lactis* ssp. *lactis*	2	0.93	0.93	0.92
- 25% + KCl	*L. lactis* ssp. *lactis*	1	0.93	0.94	0.93
- 25% + KCl	*L. lactis* ssp. *lactis*	2	0.94	0.92	0.91
-55%	*L. lactis* ssp. *lactis*	1	0.94	0.95	0.92
-55%	*L. lactis* ssp. *lactis*	2	0.94	0.93	0.92
- 55% + KCl	*L. lactis* ssp. *lactis*	1	0.93	0.94	0.93
- 55% + KCl	*L. lactis* ssp. *lactis*	2	0.92	0.94	0.90
Full Sodium	*L. lactis* ssp. *cremoris*	1	0.93	0.95	0.93
Full Sodium	*L. lactis* ssp. *cremoris*	2	0.94	0.94	0.92
-25%	*L. lactis* ssp. *cremoris*	1	0.94	0.95	0.94
-25%	*L. lactis* ssp. *cremoris*	2	0.93	0.94	0.92
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	0.93	0.93	0.93
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	0.95	0.94	0.93
-55%	*L. lactis* ssp. *cremoris*	1	0.95	0.95	0.94
-55%	*L. lactis* ssp. *cremoris*	2	0.93	0.94	0.92
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	0.94	0.93	0.93
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	0.94	0.93	0.92

A.1.3: pH

Table 8: pH (data for figure 8)

			Days														
Salt System	Starter	Rep.	0	28	53	88	94	122	136	143	152	161	170	214	227	241	262
Full Sodium	*L. l. l.*	1	4.59	4.78	4.88	4.83	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Full Sodium	*L. l. l.*	2	4.80	4.79	4.89	4.96	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
-25%	*L. l. l.*	1	4.70	4.75	4.90	4.81	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
-25%	*L. l. l.*	2	4.73	4.77	4.72	4.73	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
- 25% + KCl	*L. l. l.*	1	4.65	4.76	4.83	4.80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
- 25% + KCl	*L. l. l.*	2	4.81	4.79	4.86	4.82	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
-55%	*L. l. l.*	1	4.74	4.67	4.51	4.63	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
-55%	*L. l. l.*	2	4.83	4.82	4.74	4.79	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
- 55% + KCl	*L. l. l.*	1	4.77	4.77	4.99	4.92	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
- 55% + KCl	*L. l. l.*	2	4.84	4.81	4.71	4.78	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Full Sodium	*L. l. c.*	1	4.80	4.69	4.90	4.97	4.92	5.18	5.24	5.26	5.29	5.28	5.33	5.15	5.23	5.20	5.33
Full Sodium	*L. l. c.*	2	4.59	4.79	4.89	4.88	4.93	5.24	5.35	5.37	5.40	5.40	5.45	5.20	5.19	5.25	5.29
-25%	*L. l. c.*	1	4.73	4.68	4.94	4.82	4.78	5.21	5.30	5.31	5.32	5.33	5.38	5.27	5.18	5.23	5.32
-25%	*L. l. c.*	2	4.70	4.77	4.79	4.76	4.78	5.26	5.46	5.48	5.48	5.45	5.50	5.31	5.23	5.27	5.35
- 25% + KCl	*L. l. c.*	1	4.81	4.78	5.00	4.91	4.83	5.22	5.38	5.38	5.42	5.39	5.44	5.20	5.10	5.21	5.24
- 25% + KCl	*L. l. c.*	2	4.65	4.81	4.82	4.80	4.87	5.23	5.40	5.41	5.44	5.46	5.52	5.18	5.14	5.19	5.22
-55%	*L. l. c.*	1	4.83	4.79	4.81	4.80	4.74	5.20	5.34	5.34	5.36	5.32	5.37	5.04	5.07	5.21	5.26
-55%	*L. l. c.*	2	4.74	4.79	4.81	4.75	4.80	5.33	5.43	5.44	5.47	5.41	5.46	5.11	5.10	5.24	5.29
- 55% + KCl	*L. l. c.*	1	4.84	4.76	4.58	4.70	4.87	5.09	5.25	5.26	5.28	5.30	5.35	5.27	5.24	5.18	5.30
- 55% + KCl	*L. l. c.*	2	4.77	4.80	4.90	4.84	4.80	5.22	5.39	5.39	5.36	5.35	5.40	5.33	5.22	5.16	5.31

N/A: Not applicable; *L. l. l*: *L. lactis* ssp. *lactis*; *L. l. c.*: *L. lactis* ssp. *cremoris*

A.1.4: Gross Compositional Data

Table 9: Chemical analysis (Table 5)

	Attribute						
Salt System	Starter Culture	Rep.	Fat (%)	Protein (%)	Ash (%)	Na (mg/100g)	K (mg/100 g)
Full Sodium	*L. lactis* ssp. *lactis*	1	32.0	24.69	3.26	483	43
Full Sodium	*L. lactis* ssp. *lactis*	2	33.0	23.04	3.37	518	29
-25%	*L. lactis* ssp. *lactis*	1	33.0	25.58	2.74	379	36
-25%	*L. lactis* ssp. *lactis*	2	33.0	23.19	3.01	407	29
- 25% + KCl	*L. lactis* ssp. *lactis*	1	32.5	25.41	3.40	449	273
- 25% + KCl	*L. lactis* ssp. *lactis*	2	33.0	24.03	3.57	433	312
-55%	*L. lactis* ssp. *lactis*	1	33.0	25.11	2.20	185	27
-55%	*L. lactis* ssp. *lactis*	2	33.0	23.04	2.58	217	31
- 55% + KCl	*L. lactis* ssp. *lactis*	1	34.0	25.58	3.50	247	553
- 55% + KCl	*L. lactis* ssp. *lactis*	2	33.0	24.24	3.65	255	598
Full Sodium	*L. lactis* ssp. *cremoris*	1	32.0	24.85	3.30	470	35
Full Sodium	*L. lactis* ssp. *cremoris*	2	33.0	23.25	3.37	506	27
-25%	*L. lactis* ssp. *cremoris*	1	32.0	25.11	3.19	436	32
-25%	*L. lactis* ssp. *cremoris*	2	33.0	23.68	2.95	428	31
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	32.0	25.21	3.67	442	309
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	33.0	23.51	3.24	387	300
-55%	*L. lactis* ssp. *cremoris*	1	32.0	24.85	2.45	292	42
-55%	*L. lactis* ssp. *cremoris*	2	34.0	23.19	2.54	236	29
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	32.0	25.85	3.69	250	663
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	32.0	23.68	3.66	295	625

A.2: Microbiological testing of full, reduced, and low sodium Cheddar cheese with and without potassium chloride replacement

A.2.1: Natural flora

A.2.1.1: Total plate count

Table 10: Total plate counts. Log CFU/g counts for total plate count of samples stored at 4°C (figure 9)

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	5.13	4.44	4.39	4.91	4.63	4.82	4.95	5.55
Full Sodium	*L. lactis* ssp. *cremoris*	2	5.16	5.19	4.71	5.44	5.06	5.12	5.25	5.36
-25%	*L. lactis* ssp. *cremoris*	1	4.57	3.96	3.96	4.53	4.54	4.65	4.78	5.16
-25%	*L. lactis* ssp. *cremoris*	2	4.48	3.66	3.74	4.71	4.70	4.80	4.93	5.22
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	5.10	6.17	5.38	5.72	5.61	5.63	5.63	5.57
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	5.25	5.24	5.41	5.72	5.41	5.44	5.43	5.55
-55%	*L. lactis* ssp. *cremoris*	1	4.74	5.66	5.09	5.48	5.73	5.75	5.74	5.53
-55%	*L. lactis* ssp. *cremoris*	2	4.70	4.96	4.50	5.23	5.06	5.08	5.08	5.18
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	4.37	5.59	5.40	5.74	5.60	5.61	5.61	5.60
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.16	3.66	3.44	4.55	4.63	4.65	4.65	5.04

Table 11: Total plate counts. Log CFU/g counts for total plate count of samples stored at 12°C (figure 10)

			Days									
Salt System	Starter Culture	Rep.	0	3	6	9	12	15	18	21	24	27
Full Sodium	*L. lactis* ssp. *cremoris*	1	5.75	5.88	5.72	5.06	5.51	5.41	5.26	5.24	5.23	5.22
Full Sodium	*L. lactis* ssp. *cremoris*	2	5.62	6.20	6.64	5.61	5.64	5.68	5.64	5.63	5.61	5.61
-25%	*L. lactis* ssp. *cremoris*	1	5.73	5.75	6.46	4.99	5.72	5.66	5.60	5.58	5.57	5.57
-25%	*L. lactis* ssp. *cremoris*	2	5.49	6.10	5.51	5.15	5.43	5.38	5.44	5.43	5.41	5.42
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	5.62	6.49	6.77	5.66	5.74	5.71	5.64	5.62	5.61	5.62
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	5.65	6.18	6.11	5.21	5.59	5.63	5.47	5.45	5.44	5.45
-55%	*L. lactis* ssp. *cremoris*	1	5.62	6.59	6.88	5.59	5.76	5.73	5.67	5.66	5.65	5.64
-55%	*L. lactis* ssp. *cremoris*	2	5.51	5.73	5.44	4.66	5.35	5.00	4.93	4.91	4.90	4.88
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	5.64	6.63	6.87	5.65	5.77	5.74	5.69	5.68	5.67	5.67
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	5.53	5.76	5.54	2.96	5.07	4.34	4.98	4.96	4.95	4.97

A.2.1.2: Lactic acid bacteria

Table 12: Lactic acid bacteria counts. Log CFU/g counts of lactic acid bacteria from samples stored at 4°C (figure 11).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	8.55	8.48	8.58	8.57	8.54	8.57	8.55	8.53
Full Sodium	*L. lactis* ssp. *cremoris*	2	8.40	8.16	8.47	8.46	8.41	8.45	8.44	8.41
-25%	*L. lactis* ssp. *cremoris*	1	8.61	8.47	8.66	8.65	8.61	8.64	8.63	8.61
-25%	*L. lactis* ssp. *cremoris*	2	8.67	8.41	8.75	8.74	8.69	8.73	8.71	8.69
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	8.34	8.50	8.34	8.33	8.29	8.32	8.31	8.28
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	8.53	8.28	8.60	8.59	8.55	8.58	8.57	8.54
-55%	*L. lactis* ssp. *cremoris*	1	8.40	8.54	8.41	8.40	8.36	8.39	8.38	8.35
-55%	*L. lactis* ssp. *cremoris*	2	8.65	8.52	8.70	8.69	8.65	8.68	8.66	8.64
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	8.52	8.31	8.58	8.57	8.53	8.56	8.55	8.52
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	8.61	8.32	8.69	8.68	8.64	8.67	8.66	8.63

Table 13: Lactic acid bacteria counts. Log CFU/g counts of lactic acid bacteria from samples stored at 12°C (figure 12).

			Days									
Salt System	Starter Culture	Rep.	0	3	6	9	12	15	18	21	24	27
Full Sodium	*L. lactis* ssp. *cremoris*	1	8.44	8.11	8.85	8.01	8.51	8.49	8.53	8.51	8.49	8.51
Full Sodium	*L. lactis* ssp. *cremoris*	2	8.51	8.03	8.59	8.68	8.60	8.55	8.54	8.52	8.51	8.52
-25%	*L. lactis* ssp. *cremoris*	1	8.47	8.13	8.66	8.30	8.54	8.50	8.51	8.49	8.48	8.61
-25%	*L. lactis* ssp. *cremoris*	2	8.56	8.47	8.69	8.68	8.63	8.58	8.52	8.50	8.49	8.51
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	8.50	8.19	8.51	8.64	8.58	8.53	8.54	8.52	8.51	8.52
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	8.56	8.52	8.53	8.64	8.59	8.54	8.58	8.56	8.55	8.56
-55%	*L. lactis* ssp. *cremoris*	1	8.53	8.13	8.64	8.64	8.64	8.59	8.55	8.53	8.52	8.54
-55%	*L. lactis* ssp. *cremoris*	2	8.57	8.33	8.57	8.66	8.64	8.59	8.60	8.58	8.56	8.58
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	8.48	8.05	8.52	8.55	8.65	8.60	8.50	8.48	8.47	8.49
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	8.50	8.43	8.51	8.35	8.61	8.57	8.52	8.50	8.49	8.51

A.2.1.3: Natural spore-former count

Table 14: Log CFU/g natural spore former counts from samples stored at 4°C (figure 13).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	5.13	3.66	1.85	1.85	1.85	1.85	1.85	1.85
Full Sodium	*L. lactis* ssp. *cremoris*	2	5.13	3.96	1.85	1.85	1.85	1.85	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	1	4.57	3.96	2.56	1.96	1.85	1.85	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	2	4.55	3.66	2.56	1.85	1.85	1.85	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	5.10	3.96	1.85	1.85	1.85	1.85	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	5.22	4.91	1.85	1.85	1.85	1.85	1.85	1.85
-55%	*L. lactis* ssp. *cremoris*	1	4.73	3.66	1.96	1.85	1.85	1.85	1.85	1.85
-55%	*L. lactis* ssp. *cremoris*	2	4.74	4.26	1.85	1.85	1.85	1.85	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	4.36	3.96	2.44	1.91	1.85	1.85	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.28	3.66	2.56	1.85	1.85	1.85	1.85	1.85

Table 15: Log CFU/g natural spore former counts from samples stored at 12°C (figure 14).

			Days									
Salt System	Starter Culture	Rep.	0	3	6	9	12	15	18	21	24	27
Full Sodium	*L. lactis* ssp. *cremoris*	1	3.99	6.15	3.96	4.78	2.56	2.13	1.85	1.85	1.85	1.85
Full Sodium	*L. lactis* ssp. *cremoris*	2	3.21	6.39	1.85	2.96	2.13	2.50	1.85	1.85	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	1	4.17	5.92	1.85	2.96	1.96	2.66	1.85	1.85	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	2	3.57	5.68	3.96	3.70	2.44	1.96	1.85	1.85	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	4.22	6.03	1.85	2.96	1.96	2.13	1.85	1.85	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	3.62	6.23	3.96	2.96	2.36	2.74	1.85	1.85	1.85	1.85
-55%	*L. lactis* ssp. *cremoris*	1	4.25	6.00	1.85	2.96	2.36	2.13	1.85	1.85	1.85	1.85
-55%	*L. lactis* ssp. *cremoris*	2	4.41	6.03	3.96	4.54	2.13	1.96	1.85	1.85	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	4.43	6.06	4.44	4.43	2.13	2.36	1.85	1.85	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.39	5.92	4.66	3.13	2.26	2.96	1.85	1.85	1.85	1.85

A.2.2: *L. monocytogenes* counts

Table 16: Log CFU/g *L. monocytogenes* counts from stage 1 (figures 15, 16).

			Days					
Salt System	Starter Culture	Rep.	0	7	14	21	28	63
Full Sodium	*L. lactis* ssp. *lactis*	1	3.70	3.18	3.10	2.89	0.59	0.30
Full Sodium	*L. lactis* ssp. *lactis*	2	4.08	3.35	3.01	2.46	0.50	0.15
-25%	*L. lactis* ssp. *lactis*	1	3.66	3.24	3.02	2.93	1.30	0.30
-25%	*L. lactis* ssp. *lactis*	2	3.88	3.19	2.74	2.44	0.35	0.30
- 25% + KCl	*L. lactis* ssp. *lactis*	1	3.82	3.07	2.89	2.86	0.35	0.30
- 25% + KCl	*L. lactis* ssp. *lactis*	2	4.11	3.32	2.70	2.26	0.35	0.30
-55%	*L. lactis* ssp. *lactis*	1	3.50	3.12	2.97	2.59	0.85	0.30
-55%	*L. lactis* ssp. *lactis*	2	3.87	3.21	2.99	2.50	0.35	0.30
- 55% + KCl	*L. lactis* ssp. *lactis*	1	3.77	3.11	3.00	2.80	0.70	0.15
- 55% + KCl	*L. lactis* ssp. *lactis*	2	4.07	3.13	2.91	2.59	0.35	0.00
Full Sodium	*L. lactis* ssp. *cremoris*	1	3.95	3.15	3.02	2.82	1.57	0.30
Full Sodium	*L. lactis* ssp. *cremoris*	2	3.96	3.19	2.88	2.50	0.35	0.30
-25%	*L. lactis* ssp. *cremoris*	1	3.85	2.98	3.03	2.65	1.09	0.30
-25%	*L. lactis* ssp. *cremoris*	2	3.95	3.05	2.82	1.96	0.35	0.30
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	3.82	3.13	2.97	2.55	1.09	0.30
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	3.97	3.10	3.14	2.74	0.35	0.30
-55%	*L. lactis* ssp. *cremoris*	1	3.64	3.10	3.05	2.70	1.12	0.30
-55%	*L. lactis* ssp. *cremoris*	2	3.74	3.15	3.00	2.56	0.35	0.15
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	3.88	3.24	2.81	2.68	0.70	0.30
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.02	3.11	3.00	2.73	0.35	0.30

Table 17: Log CFU/g *L. monocytogenes* counts from samples stored at 4°C, stage 2 (figure 17).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	5.52	5.27	5.12	5.00	5.22	4.45	4.46	4.50
Full Sodium	*L. lactis* ssp. *cremoris*	2	5.62	5.36	5.17	4.95	4.82	4.18	4.09	3.61
-25%	*L. lactis* ssp. *cremoris*	1	5.55	5.29	5.18	5.06	5.20	4.55	4.59	4.40
-25%	*L. lactis* ssp. *cremoris*	2	5.58	5.36	5.18	4.90	4.44	3.97	4.10	3.76
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	5.54	5.38	5.13	5.15	4.88	4.44	4.53	4.44
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	5.56	5.22	5.14	4.45	5.05	4.87	4.93	4.81
-55%	*L. lactis* ssp. *cremoris*	1	5.56	5.70	5.38	4.75	4.50	4.36	4.40	4.46
-55%	*L. lactis* ssp. *cremoris*	2	5.61	5.53	5.39	5.40	5.32	4.94	5.03	4.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	5.62	5.29	5.12	4.92	5.11	4.34	4.36	4.19
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	5.59	5.25	5.12	4.80	5.07	4.03	4.39	4.02

Table 18: Log CFU/g *L. monocytogenes* counts from samples stored at 12°C, stage 2 (figure 18).

			Days									
Salt System	Starter Culture	Rep.	0	3	6	9	12	15	18	21	24	27
Full Sodium	*L. lactis* ssp. *cremoris*	1	4.51	4.03	4.16	5.10	4.65	5.15	5.44	5.43	5.41	5.19
Full Sodium	*L. lactis* ssp. *cremoris*	2	4.67	3.91	4.48	5.31	4.65	4.97	5.33	5.32	5.30	5.28
-25%	*L. lactis* ssp. *cremoris*	1	4.41	3.82	3.91	4.86	4.22	4.15	4.40	4.40	4.30	4.29
-25%	*L. lactis* ssp. *cremoris*	2	4.72	4.07	4.11	5.34	4.76	5.33	5.52	5.51	5.41	4.05
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	4.73	4.16	4.13	5.44	4.35	4.90	5.11	5.10	5.08	4.52
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	4.82	4.18	4.14	4.95	4.23	4.08	4.39	4.39	4.37	4.66
-55%	*L. lactis* ssp. *cremoris*	1	4.45	4.03	4.28	4.83	4.22	4.53	5.03	5.02	5.00	5.32
-55%	*L. lactis* ssp. *cremoris*	2	4.61	4.62	4.84	5.74	4.78	5.47	5.56	5.56	5.53	5.42
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	4.77	4.30	4.02	5.75	4.74	5.41	5.60	5.59	5.56	5.28
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.72	4.22	4.03	5.17	4.58	4.87	5.05	5.04	5.02	4.52

Table 19: Log CFU/g *L. monocytogenes* counts from samples made with starter culture *L. lactis* ssp. *lactis*, stage 3 (figure 19).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *lactis*	1	4.82	4.46	3.91	3.79	3.48	3.31	3.49	3.55
Full Sodium	*L. lactis* ssp. *lactis*	2	4.84	4.29	3.74	3.82	3.61	3.46	3.48	3.53
-25%	*L. lactis* ssp. *lactis*	1	4.71	4.25	3.73	3.67	3.24	3.83	3.47	3.55
-25%	*L. lactis* ssp. *lactis*	2	4.94	4.47	3.74	3.70	3.45	3.69	3.43	3.54
- 25% + KCl	*L. lactis* ssp. *lactis*	1	4.87	4.21	3.80	3.87	3.36	3.49	3.45	3.54
- 25% + KCl	*L. lactis* ssp. *lactis*	2	4.79	4.28	3.94	3.74	3.48	3.47	3.55	3.49
-55%	*L. lactis* ssp. *lactis*	1	4.67	4.26	3.70	3.74	3.24	3.48	3.59	3.49
-55%	*L. lactis* ssp. *lactis*	2	4.81	4.31	3.88	3.84	3.57	3.52	3.55	3.61
- 55% + KCl	*L. lactis* ssp. *lactis*	1	4.83	4.48	3.86	3.65	3.49	3.50	4.04	4.02
- 55% + KCl	*L. lactis* ssp. *lactis*	2	4.95	4.48	3.91	3.79	3.54	3.57	3.96	3.97

Table 20: Log CFU/g *L. monocytogenes* counts from samples made with starter culture *L. lactis* ssp. *cremoris*, stage 3 (figure 20).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	4.90	4.42	3.80	3.74	3.44	3.45	3.51	3.58
Full Sodium	*L. lactis* ssp. *cremoris*	2	4.87	4.48	3.89	3.82	3.54	3.54	3.47	3.48
-25%	*L. lactis* ssp. *cremoris*	1	4.77	4.52	3.88	3.91	3.69	3.86	3.59	3.52
-25%	*L. lactis* ssp. *cremoris*	2	4.82	4.44	3.76	3.86	3.56	3.67	3.54	3.52
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	4.85	4.40	3.86	3.78	3.56	3.60	3.60	3.47
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	4.83	4.40	3.80	3.91	3.63	3.54	3.55	3.50
-55%	*L. lactis* ssp. *cremoris*	1	4.72	4.29	3.92	3.69	3.47	3.49	3.54	3.54
-55%	*L. lactis* ssp. *cremoris*	2	4.95	4.32	3.89	3.79	3.59	3.51	3.44	3.43
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	4.86	4.38	3.93	3.84	3.56	3.53	4.01	4.00
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	4.86	4.35	3.91	3.84	3.49	3.57	3.96	3.96

A.2.3: *B. anthracis* counts

Table 21: Log CFU/g *B. anthracis* spore counts from stage 1 (figures 21, 22).

			Days					
Salt System	Starter Culture	Rep.	0	7	14	21	28	63
Full Sodium	*L. lactis* ssp. *lactis*	1	2.54	2.18	2.70	2.65	1.85	1.85
Full Sodium	*L. lactis* ssp. *lactis*	2	2.60	2.60	2.30	2.85	1.85	1.85
-25%	*L. lactis* ssp. *lactis*	1	2.70	2.54	2.98	2.18	1.85	1.85
-25%	*L. lactis* ssp. *lactis*	2	2.30	2.93	2.18	2.00	1.85	2.00
- 25% + KCl	*L. lactis* ssp. *lactis*	1	2.40	3.00	2.00	2.30	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *lactis*	2	2.48	2.48	2.30	2.48	2.30	1.85
-55%	*L. lactis* ssp. *lactis*	1	2.65	2.98	2.30	2.85	3.66	1.85
-55%	*L. lactis* ssp. *lactis*	2	2.90	2.93	2.85	2.00	2.00	1.85
- 55% + KCl	*L. lactis* ssp. *lactis*	1	2.48	2.60	2.40	2.90	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *lactis*	2	3.24	3.13	2.30	2.40	1.85	1.85
Full Sodium	*L. lactis* ssp. *cremoris*	1	2.18	2.40	2.54	2.40	1.85	1.85
Full Sodium	*L. lactis* ssp. *cremoris*	2	2.70	2.85	2.18	2.30	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	1	2.40	3.13	2.60	2.30	1.85	1.85
-25%	*L. lactis* ssp. *cremoris*	2	2.85	2.40	2.18	2.48	1.85	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	2.30	2.81	2.18	2.85	2.65	1.85
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	2.88	2.81	2.40	2.78	2.00	1.85
-55%	*L. lactis* ssp. *cremoris*	1	2.70	2.98	2.30	2.88	4.28	2.48
-55%	*L. lactis* ssp. *cremoris*	2	2.60	3.13	3.04	2.70	1.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	2.00	2.70	2.40	2.30	2.85	1.85
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	2.40	2.81	2.18	2.18	1.85	1.85

Table 22: Log CFU/g *B. anthracis* spore counts from samples stored at 4°C, stage 2 (figure 23).

			Days							
Salt System	Starter Culture	Rep.	0	7	14	21	28	35	42	49
Full Sodium	*L. lactis* ssp. *cremoris*	1	5.40	5.27	5.09	5.73	6.00	6.00	6.03	6.01
Full Sodium	*L. lactis* ssp. *cremoris*	2	5.36	5.57	4.73	5.73	6.06	6.25	5.91	5.95
-25%	*L. lactis* ssp. *cremoris*	1	5.32	5.05	5.02	5.71	5.97	5.96	6.01	6.04
-25%	*L. lactis* ssp. *cremoris*	2	5.37	5.54	4.83	5.79	6.00	6.25	5.97	5.99
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	5.29	5.22	4.90	5.69	5.88	6.02	6.02	5.93
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	5.32	5.27	4.48	5.66	5.93	5.93	5.90	5.93
-55%	*L. lactis* ssp. *cremoris*	1	5.39	5.13	5.04	5.63	5.93	5.83	5.97	5.98
-55%	*L. lactis* ssp. *cremoris*	2	5.21	5.07	4.88	5.75	6.03	6.01	5.94	5.89
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	5.38	5.19	4.61	5.66	6.00	6.00	6.03	6.02
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	5.31	5.25	4.94	5.80	6.07	6.22	6.07	6.00

Table 23: Log CFU/g *B. anthracis* spore counts from samples stored at 12°C, stage 2 (figure 24).

			Days									
Salt System	Starter Culture	Rep.	0	3	6	9	12	15	18	21	24	27
Full Sodium	*L. lactis* ssp. *cremoris*	1	6.36	6.01	5.92	5.86	5.92	5.99	5.35	6.16	6.09	5.81
Full Sodium	*L. lactis* ssp. *cremoris*	2	6.50	6.34	5.84	5.94	5.87	5.87	5.27	6.08	6.02	5.76
-25%	*L. lactis* ssp. *cremoris*	1	6.39	6.00	5.92	5.97	5.79	5.91	5.31	6.12	6.06	5.98
-25%	*L. lactis* ssp. *cremoris*	2	6.46	6.20	5.81	5.94	5.86	5.92	5.22	6.04	5.97	6.00
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	6.35	6.03	5.85	6.00	5.83	5.94	5.26	6.07	6.00	5.75
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	6.48	6.01	5.41	5.81	5.96	5.94	5.13	5.94	5.87	5.92
-55%	*L. lactis* ssp. *cremoris*	1	6.41	5.73	6.00	5.62	5.97	6.05	5.31	6.12	6.05	5.89
-55%	*L. lactis* ssp. *cremoris*	2	6.49	6.33	5.95	6.03	5.90	5.99	5.18	6.00	5.93	5.82
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	6.43	6.24	5.81	5.98	5.98	5.88	5.38	6.19	6.12	5.86
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	6.44	6.22	5.94	6.02	6.07	5.99	5.30	6.11	6.05	5.96

Table 24: Log CFU/g *B. anthracis* spore counts from samples stored at 23°C, stage 3 (figure 25).

			Hours				
Salt System	Starter Culture	Rep.	0	8	16	24	32
Full Sodium	*L. lactis* ssp. *cremoris*	1	6.09	6.12	6.05	6.04	6.02
Full Sodium	*L. lactis* ssp. *cremoris*	2	6.06	6.08	6.13	6.11	5.98
-25%	*L. lactis* ssp. *cremoris*	1	6.20	6.12	6.03	6.05	6.06
-25%	*L. lactis* ssp. *cremoris*	2	6.17	6.03	6.15	5.98	5.97
- 25% + KCl	*L. lactis* ssp. *cremoris*	1	6.12	6.02	6.13	5.97	6.05
- 25% + KCl	*L. lactis* ssp. *cremoris*	2	6.17	6.13	6.14	6.00	5.98
-55%	*L. lactis* ssp. *cremoris*	1	6.09	6.06	6.07	6.00	5.22
-55%	*L. lactis* ssp. *cremoris*	2	6.16	6.14	6.06	6.04	5.61
- 55% + KCl	*L. lactis* ssp. *cremoris*	1	6.16	6.09	6.00	5.61	5.47
- 55% + KCl	*L. lactis* ssp. *cremoris*	2	6.20	6.07	5.98	5.81	5.17