Elimination of *Listeria monocytogenes* in a Soft Cheese, Fromage Blanc, Using Processing Methods, Formulation Changes, and Additive Bacteriocin Nisin

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Abstract

Batches of fromage blanc, a soft white cheese were prepared from whole pasteurized cow's milk. Processing and formulation methods were used in cheese making to reduce *Listeria monocytogenes* in artificially contaminated cheese. Treatments implemented included use of additional starter culture in formulation (25% more starter culture than original formulation), use of a higher temperature draining process (at 45^{o}C instead of 22^{o}C), addition of the anti-listerial bacteriocin nisin (Danisco Nisaplin) in formulation at different levels (125 ppm, 250 ppm, 400 ppm), and combinations of these treatments. Characteristics including pH, fat content, protein content, and color were evaluated for each treatment cheese.

Statistically significant differences ($p<0.0001$) were found between the population (log CFU/g) values of *L. monocytogenes* in the different treatment cheeses and control cheese. Treatments using additional starter culture or higher temperature draining alone were not successful in significantly reducing numbers of *L. monocytogenes*, but when combined, a 1 log reduction resulted. Of the different concentrations of nisin used in cheese formulation, the level of 250 ppm nisin was used in combination treatments. The treatments using 250 ppm nisin were able to reduce numbers of *L. monocytogenes* by 2 log 24h after addition. Combination treatments with 250 ppm nisin and additional starter culture in formulation reduced the level of *L. monocytogenes* by only 1 log, while combination treatments coupling 250 ppm nisin with a higher temperature draining and

treatments with 250 ppm nisin, additional starter culture, and a higher temperature draining were able to reduce the pathogen by 2 log.

There were statistically significant ($p<0.0001$) differences found between cheese treatments for values of pH, fat content, and protein content. This soft cheese could be standardized for each of these parameters by the processor before packaging and sale of cheese. There were no statistically significant ($p>0.05$) differences found between colorimetric values for different cheese treatments.

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TABLE OF CONTENTS Page

LIST OF TABLES AND FIGURES Page

LITERATURE REVIEW

Listeria monocytogenes

Characteristics of the organism and distribution

In the genus *Listeria* there are six species; *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi (18)*. Only *L. monocytogenes* and *L. ivanovii* are pathogenic, but *L. ivanovii* has been determined to be pathogenic mainly in animals, so *L. monocytogenes* will be the focus of this review *(26, 31)*. Currently there are 13 serovars of *L. monocytogenes*, of these the most common isolates correlated with human illness are types 1/2a and 4b *(26)*.

Listeria are Gram positive, non-sporeforming rods that are motile within the temperature range 20 to 25^{o}C *(38)*. In this temperature range they exhibit peritrichous flagella, with characteristic tumbling or umbrella motility *(18, 38)*. Outside of this range, motility is lost as the flagella may become inactive or are simply not assembled by the organism *(18, 38)*. The optimal growth range for *L. monocytogenes* is 30^{o}C to 37^{o}C, but this organism can survive and grow at temperatures between 1^{o}C and 45^{o}C *(38)*. The psychrotrophic nature of this organism makes it a good competitor against other microorganisms *(38)*.

The growth requirements for all *Listeria* species are similar. *Listeria monocytogenes* can grow well on many standard laboratory media including trypticase soy and brain heart infusion broth or agar *(26)*. *Listeria monocytogenes* is acid tolerant, and some strains can survive in laboratory media in pH ranges from 4.1 to 9.6 *(26)*. The

optimal pH for growth of this organism is 7.0 *(38)*. This pathogen can survive at a water activity of as low as 0.90, but exhibits maximal growth at a water activity of ≥0.97 *(31, 38)*. Studies have shown that some strains of *L. monocytogenes* can survive for some time at a lower water activity of 0.83 *(31)*. *Listeria monocytogenes* is also capable of growing in laboratory media with up to 10% NaCl *(26, 38, 39)*.

Listeria monocytogenes is facultatively anaerobic. A microaerophilic environment is preferred, but it can grow in both aerobic and anaerobic conditions *(38)*. Other important characteristics of the organism include that it is catalase positive, oxidase negative, and exhibits β-hemolysis. This organism is known to be somewhat heat resistant, but overall, standard pasteurization methods are capable of destroying *L. monocytogenes* in milk; studies have found that some strains can survive high temperature short time pasteurization processing, which involves holding milk at 71.5^{o}C for 15 seconds *(18, 25, 26)*. Hurdle treatments that combine different technologies to prevent the growth of *L. monocytogenes* in food products may actually make this pathogen more resistant to single treatments, and result in the survival of more resistant strains *(31)*. For example, heat resistance of this organism is increased as the water activity of a food is decreased *(31)*. Another example is that the organism's resistance to high salt concentrations is increased when temperature is decreased *(31)*. Research that determines new hurdle methods that successfully eliminate this pathogen is desirable *(31)*.

Listeria monocytogenes is wide spread in the environment *(38)*. This bacterium has been primarily associated with soil, but is ubiquitous in nature, found also in water, plants, and animals *(38)*. This pathogen has also been isolated from decaying vegetation,

silage, sewage, and human and animal feces samples *(26, 38)*. *Listeria monocytogenes* has been isolated from many animals, but mainly from farm animals such as cattle, sheep, goats, less frequently from poultry, and from bovine milk *(38)*. There have been few isolations of *L. monocytogenes* in wild animals such as birds, fish, amphibians, and insects *(38)*.

Listeriosis

The illness listeriosis is caused by the pathogen *L. monocytogenes* in 98% of all confirmed human cases *(26)*. The causative agents of the other cases may have been either *L. ivanovii* or *L. seeligeri*, both of which are widely accepted as being non-pathogenic to humans, although *L. ivanovii* is considered a pathogen in animals *(26, 31)*. Even among animal cases of listeriosis, about 90% of these are caused by *L. monocytogenes (38)*. It is estimated that there are over 2000 human cases of listeriosis per year that result in over 500 fatalities *(31)*.

In humans there are varying levels of severity of illness after ingestion of *L. monocytogenes* which depend on different host factors *(18)*. Listeriosis mainly affects persons of specific groups including pregnant women, the immuno-compromised, and the elderly *(18)*. Infection of *L. monocytogenes* in pregnant women can result in miscarriages and stillbirths because this organism can cross the placenta to infect unborn fetuses *(38)*. Infants may also be born infected with the pathogen, causing other complications. Overall healthy individuals have been known to contract this illness, but these cases constitute a very small percentage of the overall cases *(17)*.

The characteristics of illness for listeriosis are very different from the common symptoms associated with other forms of food borne illness. The symptoms correlated to listeriosis include fever, headache, vomiting, malaise, nervous system infections, meningitis, septicemia, and severe illness may result in death *(18, 31)*. Infected patients have described the symptoms of this illness to be much like those of flu. These symptoms result in incorrect diagnosis of illness, because the doctor may believe that the patient only suffers from flu. There are treatments available for infected patients, these include different kinds of common antibiotics, such as amoxicillin and rifampicin, both of which are effective against *L. monocytogenes (24).* The onset of illness usually takes one to two days, but in some cases has taken up to five weeks. The duration is typically one to two weeks, depending on the person's overall heath *(31)*. The mortality rate in susceptible persons with listeriosis is 20 to 25% *(17)*.

The infectious dose of *L. monocytogenes* necessary to cause illness in humans is currently unknown, and expected to vary depending on different factors. One factor is the overall health of the person ingesting the bacteria, even if there are high numbers of bacterial cells present in a contaminated food and the person is healthy, they may have no adverse effects or suffer only mild symptoms. If the person is pregnant, elderly, or immuno-compromised and consumes the same high dose, the person is expected to suffer severe symptoms that may lead to death. On the other hand, if there is a low number of *L. monocytogenes* in a food, a person that is healthy overall is not expected to show any symptoms of illness. Someone from the susceptible population is expected to show mild to severe symptoms even ingestion of with smaller numbers of the organism. The serotype and strain of *L. monocytogenes* is an important factor because they have varying

levels of virulence *(18)*. It has been suggested that for healthy individuals, a dose of less than 10^2 CFU/g of *L. monocytogenes* will not result in illness *(26)*.

Pathogenesis

Listeria monocytogenes is an entero-invasive pathogen that causes disease through several steps *(38)*. The first steps of this process include the ingestion of the organism and then penetration of the gastrointestinal lining *(38)*. Once in the blood, the bacterial cells are internalized by macrophages and begin replicating *(38)*. Inside the macrophages, the cells can be transferred to various locations within the host *(31)*. After replication, macrophage lysis occurs, releasing more *L. monocytogenes* cells into the blood causing septicemia *(38)*. These cells can then invade specific organs or again be phagocytized by macrophages in which they will replicate and cause lysis further increasing numbers of *L. monocytogenes* in the blood *(38)*. *Listeria monocytogenes* can also pass directly into adjoining non-phagocytic cells, reducing the pathogens exposure to immune responses *(31)*. This pathogen's ability to be transmitted from cell to cell allow it to evade antibodies produced by the body and make its illness unique among other food borne pathogens *(31)*. The organism can be transmitted across the placenta to infect an unborn fetus and it can also invade the central nervous system *(31)*.

The virulence of *L. monocytogenes* is multifactorial *(14)*. Listeria monocytogenes can produce the protein, internalin, which gives the organism the ability to enter human cells without being phagocytized *(31)*. Once inside the host cell, intercellular movement is made possible by *L. monocytogenes* ability to utilize the host cell's actin molecules, using the ActA protein *(31)*. This organism is β-hemolytic, producing a hemolysin

enzyme, called listeriolysin O, that causes extensive lysis of red blood cells *(38)*. The ability of the organism to produce catalase may allow it to survive in phagocytes that have antibacterial agents such as hydrogen peroxide, hydroxyl radical, or hypochlorous acid. The enzyme catalase breaks down these toxic substances preventing the destruction of bacterial cells.

Related foods and outbreak

Several different foods have been correlated with *L. monocytogenes* as the agent of food borne illness. This organism was first linked to food borne illness through contaminated coleslaw in 1981 *(31, 38)*. There were 41 cases involving 34 infants and seven adults, of these 15 infants and two adults died *(38)*. In this case the cabbage used to make the coleslaw was fertilized with manure that was contaminated with *L. monocytogenes (38, 44)*. Some of the other foods correlated with outbreaks include produce items such as celery, tomatoes, and lettuce, cheese and other dairy products, chicken salad, tuna fish, raw milk, and hotdogs *(18, 48)*. Of all the mentioned foods in which outbreaks of illness have been reported, dairy products with an emphasis placed on soft cheeses is one of the most important foods correlated with the pathogen and food borne illness *(18, 37, 45, 48)*.

Pregnant women, immuno-compromised individuals, and the elderly are advised to avoid soft cheeses because they been correlated with harboring *L. monocytogenes (17, 18, 45)*. The higher incidence is due to the perfect growth conditions for *L. monocytogenes* within the soft cheeses and also because some soft cheeses are made using raw milk with an aging period that may not sufficiently eliminate the organism.

The serotypes of importance are *L. monocytogenes* 1/2a, 1/2b, and 4b, each of which have been linked to food borne outbreaks *(18, 26, 29)*. Currently 4b has been correlated with the highest number of food borne outbreaks *(26)*. It is believed that this serotype has a greater virulence than the other serovars *(26)*.

Presence and control in the processing environment

Since this pathogen may originate from numerous sources, it may be nearly impossible to prevent contamination in some areas of food processing facilities *(17)*. Workers may transport this pathogen into the processing environment on their shoes from the outdoor environment. This could results in tracking the organism to many locations within the building. Foods entering the facility may already contain *L. monocytogenes*, and processing these ingredients may lead to contamination of processing surfaces and any food contacting these surfaces later may become contaminated *(17)*. An example is raw milk coming into a dairy processing plant. Contamination by *L. monocytogenes* in raw milk may come from the soil, air, or animal (cow, goat) udders *(17)*. Studies have shown that two to four percent of all raw milk in the United States contains low levels of *L. monocytogenes (38)*. After pasteurization, the milk should no longer harbor *L. monocytogenes*, but the handling of raw milk before pasteurization is very important for reducing the probability of contamination of different areas within the processing facility.

When this organism is introduced to equipment and preparation surfaces in a processing facility, it may survive and multiply *(17, 44)*. This may lead to subsequent contamination of foods during and after processing procedures *(17, 44)*. There are several methods currently used in food processing plants to lower numbers and/or

eliminate any *L. monocytogenes* that may be present. These methods include sanitation plans for equipment, processing surfaces, in the food itself, and in the immediate environment using sanitizers and anti-microbials. Most processing facilities must use a hazard analysis critical control point (HACCP) plan along with using good manufacturing practices (GMP's) and perform adequate sampling for *L. monocytogenes* along many points in the food processing procedure *(3)*. Microbiological sampling of batches of the final product is also mandatory *(3)*. The entire plant must be monitored for presence of *Listeria* species *(3)*. Even with rigorous sampling plans in place, the time involved in microbiological testing can allow for large quantities of a contaminated food to be packaged and contaminate other surfaces before a positive *Listeria* presence is confirmed *(44)*. Some of the largest recalls in the food industry have been due to contamination by this pathogen.

In processing facilities that produce foods at risk for harboring *L. monocytogenes*, some of the processing steps may be done under refrigeration temperatures to reduce the growth of the pathogen *(22)*. *Listeria spp.* are known to survive better in moist environments, so the processing equipment and surrounding areas should be kept as dry as possible to avoid growth of this bacterium on surfaces *(22)*. Chemical agents often used in processing environments with the ability to inhibit and destroy *L. monocytogenes* include chlorine, iodine, acid anionic, and/or quaternary ammonium compounds *(22)*. Soaps, detergents, and surfactants are used in combination with these chemical agents to clean the equipment and surfaces in the processing plant *(22)*. *Listeria* species are sensitive to many antimicrobials, including chloramphenicol, macrolides, and tetracylcines *(23)*. Numerous other methods using antimicrobials have been investigated

to determine if they inhibit *L. monocytogenes (2, 8, 35, 44)*. These include ethylenediaminetetraacetic acid (EDTA), lysozyme, and lactoferrin which are often used in different combinations with other treatments to further inhibit *Listeria* in processing environments *(8)*.

Regulatory status of Listeria monocytogenes

Both the United States and United Kingdom currently follow a zero tolerance policy for *L. monocytogenes* in ready-to-eat foods, due to its ability to cause severe illness and sometimes death. European countries and Canada have established legal limits (a set tolerance of <100 CFU/g) on the level of this organism in food products, but have not yet enforced the same strict policy as the United States *(26, 31)*. Many of these countries have implemented systems of quality groups or categories to determine the acceptability of products, using more rigid policies for risk foods *(26)*. The United States and United Kingdom believe that because the infectious dose for *L. monocytogenes* is still unknown, setting tolerance limits for this organism is not adequate to ensure consumer safety *(31)*. Many argue that the zero tolerance policy of *L. monocytogenes* in foods is not cost effective *(31)*. Since *Listeria spp*. are found everywhere in nature, it is extremely difficult to prevent it from contaminating processing environments and foods. Another concern is that there is currently no 100% accurate method for detecting and enumerating this organism *(31)*. Studies have found that the incidence of listeriosis is the same in countries who implement a zero tolerance policy as it is in countries that use only tolerance levels *(31)*. Regulating this organism in foods is challenging because various levels can affect individuals very differently based on their condition of health, and

decisions are sometimes made for the most susceptible populations *(31)*. Most of the population would not be adversely affected after consumption of foods contaminated with small numbers of *L. monocytogenes*. The differences in regulatory status of *L. monocytogenes* between countries causes issues with international trading of food products *(31)*.

Ready-to-eat foods are of most concern for all governments, because these products may become contaminated after heat processing is complete and consumers are not expected to further process these foods before eating. The United States' governmental policy designates *L. monocytogenes* as an 'adulterant' in foods *(26)*. Foods containing any amount of this organism are considered adulterated and are therefore unacceptable for sale. This policy results in the recall of any foods determined to contain even a single bacterial cell of this organism in 50g of a food sample *(26)*. For these reasons, when food companies test food products for this organism, usually only a presence or absence test is involved.

Soft cheeses

There are a great number of cheeses that are classified under the category of soft cheeses *(46)*. Some of the more typical soft cheeses include cottage cheese, cream cheese, ricotta, queso fresco, queso blanco, fromage blanc, brie, and camembert *(46)*. For most soft cheeses, the initial steps of the cheese making process are similar *(10, 46)*. Milk is pasteurized using standard methods, and then concentrated through acidification. During acidification, casein, the major protein in milk, is coagulated and there is a separation of curd, which is the coagulated casein, and whey, the liquid or serum portion of milk.

Acidification of milk is accomplished through one of three basic methods. The first and most common method entails adding starter cultures made up of beneficial bacteria to milk; these organisms cause coagulation of casein through fermentation of carbohydrates in milk. The two alternative methods are either the addition of casein coagulating enzymes or food-grade acidulants. After the acidification step, the resulting curd and whey is separated through a draining process, normally achieved by transferring all the solids into sterile cheese cloth and hanging to allow liquid to drain out. After this point further processing will be different between different types of soft cheeses. The curd may be pressed to eliminate more liquids from the curd. The cheese may be shaped for packaging. Some soft cheeses have casing or a rind applied to the surface after pressing and shaping. Other cheeses may be mold ripened to add flavor. A number of soft cheeses are aged to concentrate and create flavor compounds and this process can include many variables such as time and storage conditions. Cheeses made from raw milk are normally aged for a period of six months or more to ensure that any pathogens that contaminated the raw milk are eliminated. In order to sell cheeses made from raw milk in the United States, the FDA requires this minimum six month aging period *(37)*. This law is currently under review because some pathogens, including some strains for *L. monocytogenes*, can survive in aged cheeses past the six month period *(37)*. At any point in the cheese making process methods may be applied to prevent and control pathogens and spoilage organisms in the product.

Many cheeses have standards of identity through the United States FDA's Code of Federal Regulations or internationally through Codex Alimentaris *(15, 49, 50)*. These standards of identity state the allowable ingredients and expected characteristics (fat

content, moisture content, etc.) in foods. Some soft cheeses are considered specialty products and these cheeses do not have standards of identity. Most cheeses have set standards of identity that allow for a multitude of additional ingredients to be added, which allows many soft cheeses to be categorized as specialty type cheeses in which companies can manipulate the formulation to create a unique product.

Listeria monocytogenes **and soft cheeses**

Presence in dairy products

There are two main microbiological issues in dairy products; these are contamination by pathogenic microorganisms that may cause illness in consumers and presence of spoilage microorganisms that shorten the shelf life of the product. Pasteurization and other control methods such as temperature during storage and modified packaging have been used in the past to extend the shelf life and eliminate pathogens *(1)*. Two of the most troublesome spoilage bacterial species in milk include *Pseudomonas* and *Enterobacter,* both of which are Gram negative; molds are the other problematic spoilage microorganism *(1)*. HTST pasteurization is the most common form of heat processing utilized for milk. HTST pasteurization entails holding milk at 71.5^{o}C for 15 seconds *(25)*. HTST pasteurization should destroy all pathogenic bacteria, but many studies have found that several different bacterial species, pathogenic and non-pathogenic have been able to remain viable after HTST pasteurization *(1)*. Another pasteurization method, Ultra High Temperature (UHT) pasteurization, is used when a sterile, shelf stable product is desired, in this process the milk is held for one to two seconds at 135^{o}C *(1)*. Usually pasteurization is combined with many other basic practices

to ensure a safe product, including hygiene and sanitation programs, and the implementation of a HACCP system and GMPs *(1)*.

Listeria monocytogenes is the pathogen of most importance in dairy products, because of many factors, including its presence in processing facilities, its ability to survive control procedures, and the severe illness it causes in select groups of people. Other pathogens associated with dairy products, specifically cheese, are *Escherichia coli O157*, *Bacillus cereus*, *Brucella melitensis*, *Staphylcoccus aureus* and *Salmonella spp*. *(25)*. In dairy products, *L. monocytognes* is most often correlated with causing illness in consumers from consumption of soft cheeses. This often occurs because of post pasteurization contamination of milk or curd within the processing environment.

The serotypes of *L. monocytogenes* most correlated with incidence of food borne illness from soft cheeses are 1/2a, 1/2b, and 4b *(29)*. A study of 19 soft and semi-soft cheeses found that these serotypes represented 97% of the *L. monocytogenes* found in the cheeses *(29)*.

Outbreaks

Many outbreaks of illness from consumption of soft cheeses in which *L. monocytogenes* has been determined to be the causative agent have been reported, but it is estimated that there are many more cases that go unreported in individuals who are not severely affected by the illness or may not have access to heath care facilities *(25)*.

Food borne illness is most often correlated with one of the following three situations: first, when cheese is made from un-pasteurized milk, second, when milk for cheese making has not undergone adequate pasteurization, and lastly, when

contamination has occurred after the pasteurization step *(25)*. Contamination of milk or cheese during processing can occur whether the milk has been pasteurized or not, so the dairy industry has currently been focusing research on preventative and control measures for these post-pasteurization steps.

In the United States in 1985, there were over 142 reported cases of illness caused by *L. monocytogenes* in commercial Mexican style soft cheese produced by Jalisco Products, Inc. *(11, 25, 28)*. The contaminated cheese was distributed under three different brand names over 16 states, resulting in a multi-state recall *(11)*. Of the 142 cases, 48 resulted in death *(11)*. Another outbreak of listeriosis correlated with Mexican style soft cheese in the United States occurred between 2000 and 2001 *(13)*. Investigations of this outbreak determined that the cheese was noncommercial and homemade from contaminated raw milk *(13)*. There were a total of eleven confirmed cases; ten were pregnant women and one was an immuno-compromised elderly male *(13)*. Among the ten pregnant women who were infected, there were five resulting stillbirths, three premature deliveries, and two infected newborns *(13)*. The outbreaks involving Mexican style soft cheeses were homemade by individuals and sold to restaurants *(12, 17)*. These cheeses have been cited as being made under unacceptable conditions and resulted in cases of illness due to contamination by *L. monocytogenes (12, 17)*. Between 1983 and 1987 there were over 122 reported cases of listeriosis from commercial brie cheese in Switzerland *(7, 25)*. *Listeria monocytogenes* has caused illness in persons consuming soft cheeses made with pasteurized milk and in soft cheeses that have been made with unpasteurized milk but have not undergone an aging period *(17)*.

Control of Listeria monocytogenes in soft cheeses

The intrinsic characteristics of soft cheeses are perfect for *L. monocytogenes* growth because they are slightly acidic, have high moisture content and high water activity, and have high fat content which can play a protective role for the organism against control treatments, and also because they contain high amounts of available nutrients. Because this pathogen can grow at lower pH values, like those in cheese, and at refrigeration temperatures at which the cheese will be stored, many competitive microorganisms will be eliminated.

There are several basic techniques dairy processors apply to prevent and eliminate *L. monocytogenes* in their products. These techniques include sanitation and sampling procedures within the processing environment, good hygiene practices by employees, and safe handling of ingredients for cheese making and of milk before and after pasteurization. Other preventative and control methods involve changes in the processing and formulation of a cheese. Some of these preventative measures include a heat treatment, such as a heated draining process, further increasing of acidity, lowering the water activity by addition of salt, and/or the use of antimicrobial chemicals in the product formulation. Many processors use hurdle technologies in which they combine control treatments to further ensure the safety of the final product for consumers.

Pasteurizing milk following regulatory time and temperature guidelines before cheese making can eliminate *L. monocytogenes* from contaminated raw milk *(1, 25)*. Soft cheeses may be made by several techniques *(10, 46)*. A general process for making soft cheese entails warming pasteurized milk, adding starter culture, and then allowing the

mixture to sit at room temperature for several hours. During this time fermentation occurs resulting in separation of curd and whey. The cheese is then drained in cheese cloth for a predetermined number of hours before it is ready to be packaged and refrigerated. Some soft cheeses may undergo further processing depending on the company or cheese type. Studies have shown that in most cases in which food borne illness occurred, the contamination of cheese with *L. monocytogenes* happened after the milk had been pasteurized *(22)*.

One study by Leuschner and Boughtflower (2002) found that if the milk was contaminated with *L. monocytogenes* before the cheese making process began, it could survive the process and be present in the cheese at constant concentrations for up to four weeks. This indicates that not only is the pathogen present in the cheese when processing is completed and ready for consumption, it could contaminate all the equipment and surfaces with which it comes into contact *(27)*. This study found that when the cheese draining temperature was elevated from room temperature (22^{o}C) to 45^{o}C, *L. monocytogenes* was unable to grow *(27)*.

Another control treatment for *L. monocytogenes* is to increase the acidity of the cheese. Some serovars of L. monocytogenes can survive and even grow at pHs of as low 4.1, usually a pH of lower than 5.0 places strain on the growth of this organism *(26)*. If this treatment was coupled with other control measures the level of *L. monocytogenes* surviving and growing in the cheese may be even further reduced or result in elimination of the pathogen. Lowering the pH in cheese can be achieved by using an additional amount of starter culture in milk during the fermentation step in cheese making *(41)*. Another method of lowering the pH in cheese is to directly add acids, such as lactic acid,

to the cheese or milk, the advantage of this method is that you can easily control the level of acidity. The disadvantage of this method is the resulting changes in the aroma and flavor components of the cheese. Directly adding acids may change the texture and mouth feel of the final cheese product as well, and the result may be an entirely different cheese. Both of these methods, using additional starter culture or by the addition of lactic acid, are considered natural methods *(30, 41)*. The use of additional starter culture in cheese making has previously shown to result in the inhibition of *L. monocytogenes* through the higher production of lactic acid by the lactic acid bacteria present in the starter, which increases the overall acidity of the cheese *(30, 41)*. In a preliminary study, additional starter culture (0.25g additional to the standard of 1.00g) was implemented in cheese making. The result was a reduction of greater than one pH decimal unit, from 4.67 to 4.56. This lower pH should more successfully inhibit the growth of *L. monocytogenes* than in cheese made with a normal amount of starter culture *(30, 41)*. This preliminary work also found that using additional starter culture to lower the pH of the cheese did not affect the textural, flavor, or aroma components of the final product enough for a panel to determine a difference from a control cheese in sensory evaluation.

The anti-listerial bacteriocin, nisin, has been shown to inhibit the growth of *L. monocytogenes* when applied in foods *(1, 35, 37, 40)*. Nisin may be added to milk as an additive in the commercial grade formulation or lactic acid bacteria that produce this bacteriocin may be added along with the starter cultures to the milk at the start of the cheese making process *(1, 35, 40)*. High levels of this bacteriocin have been shown to completely eliminate *L. monocytogenes* in soft cheeses within periods as short as 24h *(37)*.

The food industry has recently been experiencing a trend, brought on by consumers, to make more products which are produced using natural methods *(21, 42)*. The popularity of organic foods has helped to spur this trend. Consumers are becoming more health conscious and some have begun to voice their concerns about man-made chemicals that may be used in foods to prevent growth of pathogens. The control methods discussed, if implemented in cheese making, would allow for the final product to be labeled as an 'all natural' or 'naturally produced' food.

Anti-listerial bacteriocin, nisin

Characteristics

The bacteriocin, nisin, has been shown to be effective in the inhibition of growth and lowered survival of several food borne spoilage organisms and pathogens *(33)*. The compound nisin is produced by several subspecies of the lactic acid bacterium *Lactococcus lactis,* such as ssp. *cremoris* and ssp. *lactis (4, 5, 34, 36)*. Nisin is specific for antimicrobial action against Gram positive organisms, but has been known to affect Gram negative bacteria that have damaged outer membranes *(4)*. A study challenged the Gram negative bacterium *Salmonella* first with the chelating agent EDTA, which is capable of weakening cell membranes, then with nisin, they found that nisin was bactericidal against this organism when accompanied by a chelating agent *(43)*.

Nisin is a proteinaceous toxin made up of a chain of 34 amino acids with a molecular weight of 3500Da *(4, 33)*. Nisin has been shown to be heat and acid stable *(33)*. There are two known forms of nisin, A and Z *(4, 33)*. These two forms only differ by one amino acid, the 27^{th} on the chain, in nisin A this amino acid is histidine and in

nisin Z it is asparagine *(4)*. Nisin Z is normally used over nisin A in food studies because nisin Z has higher solubility and diffusion characteristics *(4)*. A powdered version of nisin is commercially available as an additive for foods, but contains nisin A *(4)*.

Anti-bacterial mechanism

Nisin uses a very specific mechanism to destroy Gram positive bacteria. Nisin will bind to a cell membrane lipid and incorporate itself as a pore into the membrane, which results in disruption *(9)*. The mechanism is due to the overall cationic nature of nisin *(9)*. When nisin is in the presence of negatively charged phospholipid groups on the bacterial cell membrane, nisin will change conformation to project its many positively charged side chains on the amino acid chain towards the membrane to begin interaction and integration *(9)*. It works much like a detergent, solubilizing the membrane, resulting in leakage of important cellular molecules *(9)*. Nisin is not able to destroy normal Gram negative bacteria because of its large size and ring conformation, which is caused by the presence of thioester amino acids *(9)*. If the membrane of a Gram negative cell is damaged, by some chemical method, such as use of a chelating agent, then the nisin can further disrupt the membrane leading to destruction of the bacterial cell *(4, 43)*.

Regulations concerning use of nisin in foods

There are regulations set in place by the FDA and USDA for the levels of nisin allowed in different foods *(47)*. In cheeses, the FDA allows a maximum of 250ppm of nisin in the finished product *(50)*. Ready-to-eat meats may harbor the pathogen *L. monocytogenes* so concentrations of acceptable nisin in these products have also been

suggested by government agencies. The acceptable levels in meats, mostly for meat and poultry soups and slurries, is no greater than 5ppm in the product's formulation *(47)*. The USDA also allows a maximum of 600ppm in sauces (made under FDA regulations) that are used with meat and poultry *(47)*. Nisin is currently utilized in many meat products, but has not yet been widely used as an antimicrobial agent in cheese making.

Nisin in soft cheeses

Nisin is produced by lactic acid bacteria that can act as starter cultures capable of acidifying milk through acid production during fermentation of carbohydrates in milk to result in curd that may be separated and further processed to become cheese. The production of nisin by lactic acid bacteria is a completely natural process, as its production is a mechanism these bacteria use to help survive against other Gram positive organisms competing for nutrients. As these lactic acid bacteria are fermenting carbohydrates from milk, growing and dividing, nisin compounds are released into their surroundings. When scientists discovered that nisin and other bacteriocins could be bactericidal to problematic food borne pathogens, research testing the antimicrobial mechanism and safety for human consumption has been of interest *(36)*.

Nisin's ability to destroy *L. monocytogenes* is important because this organism is a problematic pathogen in cheeses and has caused issues in food safety of dairy products *(6, 16)*. *Listeria monocytogenes* has been isolated from raw milk, but in most cases, it contaminates either the milk or cheese during processing following the pasteurization procedure *(36)*. Many studies have established nisin's ability to act as an antimicrobial against this Gram positive pathogen, and many other microorganisms *(4, 6, 19, 20, 34,*

36). Even though nisin could be used in cheese formulations as a preventative method against this pathogen, it is not currently being applied in soft cheese production by a large number of companies. Nisin is also effective against the Gram positive organism, *Clostridium botulinum*, a food borne pathogen that was mainly correlated with causing a gas blowing defect in cheeses rather than causing wide spread infections *(32)*. Other genera of Gram positive microorganisms that are negatively affected by nisin include *Bacillis, Lactobacillus, Staphylococcus,* and *Streptococcus (33)*.

The commercial grade additive form of nisin is a powder containing 2.5% nisin. Nisin used for this powder formulation is obtained from lactic acid bacterial cells. Once nisin is produced, it is separated through centrifugation or filtration. The lactic acid bacterial cells are then inactivated and the molecules of nisin are reattached to these unviable cells. This solution is dried and stabilizers are added to the powdered product. In this form the nisin is still considered an all natural additive by the FDA. It is also listed as a generally recognized as safe additive for foods.

Processors add concentrations of nisin to foods in parts per million (ppm) or Standard International Units (IU). This powder can be added directly to a semi-aqueous product and the concentration of nisin at addition is known with a simple calculation. In cheese making, if the nisin is added to the milk with the starter cultures before setting, some of the nisin could be lost during processing, so there are several methods established for quantifying nisin in food products. A way to avoid the loss of nisin is to add it after the draining process, the problem with addition at this point is the possibility of overgrowth of the target pathogen during setting and draining to numbers impossible

for the nisin to be adequately effective. Powdered nisin has also been used as an antimicrobial in meat slurries and other meat products.

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Chapter 1

Elimination of *Listeria monocytogenes* in a Soft Cheese, Fromage Blanc, Using Processing Methods, Formulation Changes, and Additive Bacteriocin Nisin

INTRODUCTION

Listeria monocytogenes causes the food borne illness listeriosis *(2, 4, 7, 17, 20)*. In the United States, there are over 2,500 cases of this illness reported per year with around 500 of these cases resulting in death *(20)*. The cost related to food borne *L. monocytogenes* illness is estimated to be $2.3 billion per year *(20)*. Since this pathogen can cause severe illness and even death in certain individuals, the United States government has implemented a zero tolerance policy for *L. monocytogenes* in ready to eat foods. This policy also encourages processors to use anti-listerial treatments in their products to avoid recalls. Even with these strict laws in place requiring ready to eat foods to be free of *L. monocytogenes*, outbreaks still occur in many foods, with one of the important risk foods being soft cheeses *(2-4, 6, 15)*.

Soft cheeses may become contaminated with *L. monocytogenes* at many different points in the cheese making process *(19)*. *Listeria monocytogenes* is present in soil, air, and is also naturally found on cows and goats from which milk for dairy products is obtained. This pathogen is often found in dairy processing environments and can originate from many different entry routes *(6)*. Once in the processing environment, *L. monocytogenes* can survive and sometimes grow on equipment and preparation surfaces which could contaminate food processed in these areas *(6, 19)*. In some cases the milk is

contaminated with *L. monocytogenes* after pasteurization and *L. monocytogenes* may survive the cheese making process *(11)*.

Recently, natural foods are becoming popular with consumers. Consumers want to purchase products that are guaranteed to be safe but have an 'all natural' label *(1, 10, 12)*. In this study, natural methods of changing the cheese making process and formulation of the cheeses will be used with the aim of eliminating this pathogen.

The Centers for Disease Control and Prevention (CDC) and the FDA have set a goal to reduce the number of cases of listeriosis by 50% by the year 2010, so new methods of reducing and eliminating *L. monocytogenes* survival in foods are needed to help meet this goal *(2)*. It is important for the food industry to find and use anti-listerial methods given the potential of this pathogen to cause severe illness and death and its ability to invade processing environments to contaminate food.

OBJECTIVES

- To use processing and formulation methods in cheese making to obtain a reduction in numbers of *L. monocytogenes*

- To determine which single method inhibits the greatest number of *L. monocytogenes* in the soft cheese, fromage blanc

- To determine the reduction of *L. monocytogenes* by different treatment combinations in the soft cheese, fromage blanc

MATERIALS AND METHODS

Cultures and culture maintenance

Listeria monocytogenes serotypes used included 1/2a (FSL C1-056), 1/2b (FSL R2-50), and 4b (FSL J1-110) obtained from the Weidmann collection from Cornell University *(9)*. These cultures were stored in blood heart infusion broth with 15% glycerin (Acros) in an ultra-low freezer set to -70°C. To revive cultures, 1.0 µl of each *L. monocytogenes* serotype, 1/2a, 1/2b, and 4b, was added to 30ml of tryptic soy broth (Difco) supplemented with 0.6% yeast extract (TSBYE) (Fisher Biotech) using 1.0 µl loops (Fisher Scientific). This cocktail of the three *L. monocytogenes* serotypes was incubated for 24h at 35°C before 3.0 µl was transferred to fresh TSBYE and incubated for an additional 24h. An inoculum of 3.0 µl was transferred as described above to 30 ml fresh TSBYE three times before the culture was used. Streak plates and spread plates were made onto Modified Oxford Agar (Difco) and these plates were incubated for 48h. Standard plate counts were used to verify the level of *L. monocytogenes* contained in the stock culture. Isolated colonies from streak plates were morphologically examined and a typical colony was analyzed using an API Listeria (BioMerieux) to confirm the colonies as *L. monocytogenes*.

The *Lactobacillus sake* (ATCC 15521) culture used for nisin quantification was obtained from Dr. T. Montville from Rutgers University. The cultures were stored in blood heart infusion broth with 15% glycerin in an ultra-low freezer (approx. -70° C). *Lactobacillus sake* was revived in MRS broth (Oxoid, Hampshire, England) at 35°C before being incorporated into MRS agar.

Cheese making

The cheeses were made in the Food Science and Technology building at the Blacksburg campus of Virginia Tech. Cheese was made in a laboratory designated for *L. monocytogenes* (CDC-Biosafety Level II). Whole, pasteurized milk for cheese making was purchased from a local grocer. Butter muslin cheese cloth was obtained from the New England Cheese Making Company (Ashfield, MA). Liquid rennet (Chymostar Classic) and starter cultures (CHOOZIT MM 101 LYO 50 DCU Cheese Cultures) were obtained from Danisco. To make this cheese, 3.785 L (one gallon) of pasteurized whole milk was poured into a 5 L sterile polypropylene container (Nalgene) and heated to 30^{o}C in a large water bath over 20 to 30 minutes. Temperature readings were measured with a calibrated digital thermometer (Omega). One packet (1 g) of starter culture consisting of *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris,* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* was added to the heated milk. Liquid rennet (0.3 ml) was dissolved in 180 ml sterile water then added to the milk. The milk, starter culture, and rennet solution were mixed thoroughly for one minute, covered, and allowed to set at 22^{o}C for 12h. After 12h, the curd was ladled into a steam sterilized polypropylene container through one layer of previously steam sterilized cheese cloth (New England Cheese Making Supply Company). The corners of cheese cloth were tied together around a steam sterilized metal bar and drained, covered, for 6h. After draining, the cheese was packaged into commercially obtained 16 oz plastic containers with lids that were sterilized with chlorine (200 ppm) and then stored in a 4^{o}C refrigerator.

Processing and formulation changes were implemented to create different treatments, which are described in the following sections.

When a batch of cheese was inoculated with *L. monocytogenes*, the culture was added to milk after heating and addition of starter cultures and rennet to mimic the most likely point of contamination in an industry processing environment. *Listeria monocytogenes* inoculum for cheese making was prepared through a three day fresh nutrient exchange with verification step using API Listeria. An inoculum of 3.8 ml of *L. monocytogenes* in TSBYE was added to milk to achieve 6 log CFU/ml milk.

Treatments in soft cheeses

Cheese Treatment	Un-inoculated Control	Inoculated with 6 log *Listeria monocytogenes*
Made with standard formulation and processing procedure	X	X
Made with additional starter culture (25% more) in formulation	X	X
Made with a higher draining temperature (45^{o}C)	X	X
Made with 125 ppm Nisaplin in formulation	X	X
Made with 250 ppm Nisaplin in formulation	X	X
Made with 400 ppm Nisaplin in formulation	X	X
Made with additional starter culture (25% more) in formulation and higher draining temperature (45^{o}C)	X	X
Made with 250 ppm Nisaplin and additional starter culture (25% more) in formulation	X	X
Made with 250 ppm Nisaplin in formulation and higher draining temperature (45^{o}C)	X	X
Made with 250 ppm Nisaplin in formulation and additional starter culture (25% more) in formulation and higher draining temperature (45^{o}C)	X	X

Use of additional starter culture in formulation

For this treatment 25% more starter culture was added to heated milk at the start of cheese making. A total of 1.25 g of starter culture was added to 3.785 L milk.

Application of a higher draining temperature

For the treatment of higher draining temperature, the cheese was made following the standard cheese making procedure except that the curd was drained in a 45^{o}C incubator.

Use of the bacteriocin nisin in formulation

For these treatments additive grade powdered Nisaplin was used in formulation. Nisaplin was added and mixed into cheese after cheese making was completed. Preliminary studies were done to determine the optimum concentration of nisin to use in further hurdle treatments. Three concentrations were evaluated, 145 ppm, 250 ppm, and 400 ppm. Each of these concentrations was used in cheese inoculated with 6 log *L. monocytogenes*. Standard plate counts were used to determine the survival of *L. monocytogenes* in cheese made with each level of nisin. Based on these results, one concentration was chosen for further research. The level of nisin used in cheese formulation was determined using an agar diffusion assay.

Agar diffusion assay for nisin quantification

Techniques implemented involving nisin quantification followed methods used by Rogers and Montville *(16)*. For the preliminary study, nisin standards were made for each of the three concentrations, 125 ppm, 250 ppm, and 400 ppm by adding calculated amounts of powdered Nisaplin to aqueous 10ml of 0.02 N HCl 0.75% NaCl solution. The solutions were adjusted to a pH of 5.3 using a calibrated pH meter and then steam sterilized before use in the assay. For cheese treatments, samples were prepared in a 1:1 dilution in 0.02 N HCl 0.75% NaCl before use in the assay.

For the assay, MRS agar was tempered to 45^{o}C in a water bath before *L. sake* culture in MRS broth made with 1.5% Noble agar was incorporated to a final concentration of 5 to 6 log CFU/ml. A steam sterilized metal #3 cork borer (6.8mm) was used to bore four wells into the duplicate MRS plates to hold 50 µl of nisin standard or cheese sample. Once 50 µl of the nisin standard or cheese sample was pipetted into each of the four wells on duplicate plates, the plates were incubated at 3^{o}C for 3h to 24h in preparation for the assay. Then the plates were incubated for 24h at 30^{o}C, the zone of growth inhibition (mm) was measured for at least six wells. The measurement was taken from the edge of the well to the edge of the growth.

Microbial Testing

Detection of *L. monocytogenes* followed the method used by Leuschner *(13)*. In this method, a 25 g sample of cheese was mixed with 225 ml of Half Fraser broth and incubated at 30^{o}C for 24h. From this, 0.1 ml culture was taken and added to 10ml of full

Fraser broth and incubated at 37°C for 48h. This secondary enrichment was plated unto Modified Oxford Agar (MOX) and incubated at 30°C for 48h. The plates were examined for growth indicative of *L. monocytogenes*. Confirmation was performed using API Listeria.

Plate counts were used for enumeration; a 10 g sample of each cheese was mixed with 90 ml of peptone water in a stomacher bag to give a 1:10 dilution and masticated for two minutes. Enumeration of *Listeria* was done on either duplicate MOX plates with serial dilutions. All plates were incubated at 30°C for 48h.

Analytical Tests

For each cheese treatment, the pH, fat content, and protein content were determined using the standard method described in the Standard Methods for Dairy Products *(22)*. For pH determination, a calibrated pH meter was used. To determine fat content percentage the Babcock method of fat determination was used. To determine protein content percentage the Kjeldhal method of nitrogen determination was used and protein content was calculated from values obtained.

Each cheese treatment was compared visually and with a Minolta colorimeter always in the same lighting environment. The colorimeter was used to determine the chromatistic coordinates of each sample using the L*a*b* color space. L*a*b* color space measures the lightness (L), redness (a), and yellowness (b) of a sample. The colorimeter was calibrated before each measurement using a standard color tile that was the color of the control soft cheese. The different treatment cheese samples were prepared through thorough mixing in their container. A clear Petri plate was laid over the cheese

sample and pressed flat so that a uniform amount of cheese was under the plate for accurate readings. Three measurements were taken for each cheese treatment.

Statistical Analysis

This study was of completely randomized design. The mean log survival of *L. monocytogenes* found by standard plate count on MOX plates for each treatment were compared using ANOVA and Tukey's HSD multiple comparisons if differences between means were found.

The mean pH values, fat content values, and protein content values of cheese samples for each treatment were compared by ANOVA and if differences were found between the means, multiple comparisons using Tukey's HSD were done. The mean values from color analysis were compared by ANOVA and also Tukey's HSD multiple comparisons if differences were found.

All of the statistical analysis was done using JMP 5.0, by SAS Institute.

RESULTS & DISCUSSION

The soft cheese chosen for this study was fromage blanc, because it is representative of many other soft cheeses, is easy to process, and is relatively inexpensive to make. *Listeria monocytogenes* was chosen because it has been repeatedly linked to food borne illness with soft cheeses *(2, 3, 20)*. A cocktail of three serotypes of *L. monocytogenes*, 1/2a, 1/2b, and 4b, were chosen for artificial contamination of cheese based on their prevalence in outbreaks associated with soft cheeses *(14)*. For enumeration of this pathogen in cheese samples, MOX was chosen for its selectivity for *L. monocytogenes*.

Results of Microbiological analysis of cheese treatments

Confirmation of control levels of L. monocytogenes

The inoculation level of L. monocytogenes in milk was approximately 6 log. To confirm this inoculation level of the pathogen in cheese, control treatments made using the standard formulation and processing methods were sampled directly after cheese making was complete and were determined to have an average survival of 6.4 log *L. monocytogenes*. The range of *L. monocytogenes* survival in control treatments was 6.3 to 6.5 log CFU/g.

Reduction of L. monocytogenes in treatment cheeses

Statistically significant ($p<0.0001$) differences were found between the population log values CFU/g for different treatment cheeses. Some treatments were able to

significantly lower the level of *L. monocytogenes* from the control treatment, while others could not. Table 1 presents the population log values CFU/g cheese of *L. monocytogenes* found for different cheese treatments, and the results of Tukey's HSD multiple comparisons of the values. Figure 1 graphically represents the population log CFU/g cheese levels of survival of *L. monocytogenes* in each treatment cheese and in the control cheese.

Results of preliminary study with Nisaplin

Preliminary experiments were preformed to determine the optimum concentration in parts per million (ppm) of nisin in formulation of cheese to use in hurdle treatments. Three different levels of commercial grade additive nisin, Nisaplin, were used in preliminary experiments, these included 125 ppm, a lower concentration than the allowable amount in soft cheeses, 250 ppm, the maximum allowable limit in soft cheeses, and finally 400 ppm, to determine whether this bacteriocin could be more effective at a level greater than the maximum allowable limit.

For all three different levels of nisin used, 125 ppm, 250 ppm, and 400 ppm, the level of *L. monocytogenes* found after 24h was on average 4.2 log CFU/g, which is over a 2 log reduction from the control treatment. Table 2 presents the population log values CFU/g of *L. monocytogenes* in cheese treatments for each of the three levels of nisin at 24h and 48h against the control treatment. There were significant differences found between survival of *L. monocytogenes* in cheeses with the 125 ppm concentration and the two higher concentrations. The survival of *L. monocytogenes* in cheeses with concentrations of 250 ppm and 400 ppm were not significantly different. The 250 ppm

nisin concentration was chosen for use in hurdle treatments because it was more effective than the 125 ppm and equally effective as the 400 ppm level. If these concentrations are equally effective, then processors should use the lower amount. The level of 400 ppm is also above the allowable limit for commercial nisin in soft cheese so this level could not currently be used by processors.

These preliminary studies had 24h and 48h sampling. The resulting survival of L. *monocytogenes* after 48h of addition of Nisaplin to cheese was not significantly different from the survival at 24h. After 24h all three different concentrations of nisin offered the same 2 log reduction. The 48h sampling was dropped for this reason and because processors would probably not be willing to do a 48h sampling because of the limited shelf life of fresh soft cheeses.

Results of single cheese treatments

For treatments with additional starter culture, a 25% increase in starter culture was chosen based on results from a preliminary study which determined that this amount significantly lowered the pH without changing the sensory acceptance of cheese. A sensory panel could not determine a difference between cheeses made with this treatment and control cheese.

Cheeses made with additional starter culture alone were unable to significantly reduce the survival of *L. monocytogenes* from the level in control cheese. The average reported count in cheeses with this treatment was 6.2 log CFU/g. The range in values of survival of this pathogen in this cheese was 6.1 to 6.3 log CFU/g.

Treatments using a higher draining temperature alone had an average reported count 6.3 log CFU/g. This demonstrates that using an elevated temperature during draining as a single treatment was unable to significantly reduce the level of *L. monocytogenes* from control treatment in this cheese. In this study, the survival of *L. monocytogenes* may not have been significantly reduced by this treatment, but growth of the organism may have been prevented. A previous study found that an elevated temperature draining at 45^{o}C was able to prevent growth, but not eliminate *L. monocytogenes (13)*.

The most successful of the single treatments implemented was the addition of the anti-listerial bacteriocin nisin in formulation. The cheese treatments using 250 ppm nisin in formulation alone resulted in over a 2 log reduction after 24h, with an average reported count of 4.1 log CFU/g. The range in values of survival of L. monocytogenes in the single nisin treatment cheeses were 4.0 to 4.1 log CFU/g. Studies have shown that the likely contamination level of *L. monocytogenes* in foods from the processing environment reach less than 1 log in the final product *(21)*. Even though this seems a low level, the infectious dose of this pathogen is unknown and as low as a few cells may be able to cause severe illness in susceptible populations, so if there is a positive result for *Listeria* presence, the food must be recalled *(15)*. The use of 250 ppm Nisaplin in formulation in this soft cheese should be able to completely eliminate *L. monocytogenes* from the final product within 24h of addition.

Effectiveness of hurdle treatments

When the use of additional starter culture and a higher draining temperature were coupled together as a treatment, the result was a log reduction of *L. monocytogenes*. The average reported count for this treatment was 5.5 log CFU/g, which was significantly different from the level of the pathogen found in the control according to Tukey's HSD multiple comparisons, as presented in Table 1. Based on the low levels of expected contamination with *L. monocytogenes*, this hurdle treatment may be able to make the finished cheese product *Listeria* free.

In the treatment coupling 250 ppm nisin with additional starter culture in formulation of cheese, a 1 log reduction in *L. monocytogenes* resulted. For this treatment, the population reported count was 5.3 log CFU/g after 24h. The coupling of additional starter culture and 250 ppm nisin in formulation seemed to lower the effectiveness of the nisin. There were statistically significant ($p<0.0001$) differences between the mean log values of *L. monocytogenes* in this treatment cheese compared to the mean log values found in all other treatment cheeses implementing nisin in formulation, including using nisin as a single treatment (Table 3).

For the treatment combining 250 ppm nisin with a higher draining temperature, a 2 log reduction in *L. monocytogenes* was seen. The mean reported count was 4.4 log CFU/g after 24h. Significant differences were not found between this treatment and the survival of *L. monocytogenes* for the treatment using nisin as a single treatment. The previous study using higher draining temperatures in soft cheese found that this treatment may not eliminate *L. monocytogenes* from cheese, but at this temperature its growth is

completely inhibited *(13)*. The cheese used in this study was drained for a total of 6h, but many similar soft cheeses may be drained for up to 12h. When this longer draining period is done at 22°C, this allows *L. monocytogenes* that may contaminate the soft cheese a wider time frame to grow and multiply. It is possible that in this situation the pathogen could grow to levels greater than that of nisin's ability to eliminate. If the higher draining temperature was used in processing of soft cheeses with a longer draining time, the level of contamination from earlier steps in cheese making could be inhibited and kept low enough that within 24h of addition of nisin, the pathogen could be eliminated.

The treatment that combined all the processing and formulation changes including a higher draining temperature, and additional starter culture and 250 ppm nisin in formulation resulted in a 2 log reduction in *L. monocytogenes* similar to the other nisin treatment combinations. The population reported count was 4.6 log CFU/g after 24h.

Based on these results, it may be assumed that although the combination treatments of additional starter culture in formulation and a higher draining temperature was able to reduce the level of *L. monocytogenes* by 1 log, this reduction is not additional to the 2 log reduction of *L. monocytogenes* resulting from 250 ppm nisin in formulation. Perhaps the combination of these treatments activates resistance factors of the organism *(15)*. It has been well established that *L. monocytogenes* may better survive individual treatments such as heat, salt, or acidity when treatments are combined *(15)*. In treatments implementing additional starter culture in formulation, a greater number of lactic acid bacteria are added to milk, therefore, more viable organisms are in competition for the fermentable carbohydrates in the milk, to survive and compete with these organisms, *L. monocytogenes* may have needed to activate resistance factors *(15)*.

Nisin diffusion assay to quantify nisin levels in cheese

Table 4 presents the inhibition diameters measured of *L. sake* on MRS agar from the nisin diffusion assay for control samples and cheese samples in the preliminary study with nisin. There were statistically significant ($p<0.0001$) differences found between inhibition zones for control samples of each concentration and the respective concentration when used in cheese. This shows that an aspect of the cheese is affecting the antimicrobial activity of nisin. Previous studies have shown that the activity of nisin is lowered when used in soft cheese, due to its high fat content *(18)*. The fat molecules in cheese can have a binding affect with nisin, but its activity is not completely inhibited *(18)*.

The mean inhibition zone for *L. sake* on MRS for the 125 ppm control was 14.0 mm and the inhibition zone was 7.2 mm when in cheese. The mean inhibition zone was 14.9 mm in the control sample and 9.2 mm in the cheese sample for the 250 ppm concentration. For the 400 ppm concentration, the average inhibition zone measured was 15.3 mm for the control sample and 9.8 mm for the cheese sample. These results show that the concentration of nisin used in soft cheeses may be able to be quantified by using this diffusion assay when the binding of nisin by fat particles is taken into account.

Results of analytical analysis

The analytical tests performed on the different treatment cheeses including pH, fat content, protein content, and colorimetric analysis were analyzed through ANOVA using the JMP 5.0 statistics program from SAS. Statistically significant differences were found

between mean values of pH, fat content, and protein content, but significant differences were not found for any of the parameters of colorimetric analysis. Table 5 presents the resulting mean values from analysis of pH, fat content, and protein content performed for all cheese treatments. Table 6 presents the resulting mean values from colorimetric analysis.

pH

There were statistically significant differences ($p<0.0001$) between values of pH recorded for the different cheese treatments. Differences were expected between treatments. Using additional starter culture in formulation should increase acidity through increasing the number of lactic acid bacteria fermenting carbohydrates in milk, resulting in a higher production of lactic acid. This was seen in preliminary experiments, but not when milk for cheese making was contaminated with *L. monocytogenes*. The lowest mean pH value (4.22) was found when additional starter culture was coupled with high draining temperature and in the treatments with only high temperature draining (pH=4.24). So the high temperature draining treatment may be lowering the pH. The mean pH value for the control cheese was 4.25. The range in mean pH values was 4.22 to 4.43.

The addition of the bacteriocin nisin in powdered form increased the pH slightly from the control treatment value. In the control treatment, the average pH value was 4.28, but the average pH value for the treatments that included nisin was 4.40. This may be attributed to the composition of the commercial grade powdered formulation of nisin

used in cheese making, which contains a minimum of 50% sodium chloride, a basic reagent that will increase the pH value of an acidic food *(5)*.

Fat content

There were statistically significant differences ($p<0.0001$) between values of fat content (percent fat) recorded for the different cheese treatments. The lowest mean value was 8.48% fat in treatment batches made with the addition of 250 ppm nisin to formulation coupled with a higher temperature draining. The highest mean fat content value was 10.68% fat in treatment batches made using additional starter culture in formulation with the combination of a higher temperature draining. The mean fat content value for the control cheese was 8.98%. No clear trends were determined between the fat content values between different treatments.

Protein content

There were statistically significant differences ($p<0.0001$) between the values of protein content (percent protein) recorded for the different cheese treatments. The lowest average value of protein content was 7.21% protein in treatments in which only 250 ppm nisin was implemented. A pattern of decreasing protein content was recorded as the level of nisin increased in cheese treatments in the preliminary nisin experiments. The highest mean value of protein content was 8.57% in control cheese batches made under normal processing and formulation methods.

The differences found in protein concentration of different treatment cheeses may be remedied in the industry through addition of nitrogen to the cheese formulation to

obtain the desired level for the final product. Many factors of milk can alter the final protein content of cheese products, including diet, season, breed, and stage of lactation of the cow from which milk was obtained so many processors currently implement methods to control the protein concentration for the final product *(8)*. A mixing process is necessary after production of soft cheeses to create a more uniform product before packaging. Processors could easily use additives to standardize the components of this soft cheese before distribution.

Colorimetric analysis

Three colorimetric parameters were measured for each cheese treatment sample, these included lightness (L), redness (a), and yellowness (b). The cheese was also visually evaluated and no differences could be distinguished between the treatment cheeses. This cheese always had a pale, light off-white appearance.

There was a slight statistical significant difference ($p=0.0426$) found between mean values of lightness, but when Tukey's HSD multiple comparisons was performed, no differences between means were found. The lowest mean value for lightness was 88.94 for the treatments combining higher temperature draining, and additional starter culture and 250 ppm nisin in formulation. The highest mean value for lightness was 90.59 for the control treatment. A high value for lightness indicates a pale, light color in cheese treatments.

There were no statistically significant differences ($p=0.1573$) found between mean values of redness in cheese treatments. The lowest mean value for redness was -1.84 for the control treatment cheeses. The highest mean value for redness was -1.61 for

the treatments combining 250 ppm nisin in formulation with higher temperature draining. Negative redness values indicate the color direction to be in the green direction.

There were no statistically significant differences ($p=0.5280$) found between mean values of yellowness in cheese treatments. The lowest mean value for yellowness was 7.04 for the treatment combining 250 ppm nisin in formulation with higher temperature draining. The highest mean value for yellowness was 7.64 in the control treatments. These results show that there may be an inverse relationship between redness and yellowness for this cheese. Positive yellowness values indicate the color direction to be in the yellow direction.

CONCLUSIONS

The level of *L. monocytogenes* contamination was very high in this cheese, at approximately 6 log per ml of milk. From a processing environment, the contamination by *L. monocytogenes* would be expected to be much lower, the FDA reports that it is expected to be less than 1 log, and the results of this study show that use of nisin should be effective in eliminating this level of the pathogen from this cheese and perhaps similar soft cheeses *(21)*. Several treatments from this study were able to reduce the level of *L. monocytogenes* by 1 log to over 2 log. The treatment of 250 ppm nisin alone in formulation was able to reduce the pathogen by over 2 log, so this treatment is recommended for processors. The treatments using 250 ppm nisin with additional starter culture and the treatment combining nisin, additional starter, and a higher draining temperature were also effective in reducing L. monocytogenes by 2 log.

Additive grade nisin, called Nisaplin, is commercially available for processors in a powdered form that is legal for use in soft cheeses at up to 250 ppm concentration in final formulation. There are many benefits for its use in soft cheeses. Nisin can specifically destroy pathogens, but not starter culture microorganisms. It is very easy to use, only a simple calculation is needed to determine the exact amount needed to add to the product to get a specific concentration. Nisin will not change the flavor or color of the cheese, which is important for specialty cheese makers *(5)*. It is also considered a GRAS additive and a natural additive, so the final product can retain an 'all natural' labeling *(5)*. Finally, Nisaplin is formulated to be acid and heat stable *(5)*.

Some smaller cheese processors may not be budgeted to use Nisaplin in their products. The treatment using additional starter and a higher draining temperature were able to reduce the level of *L. monocytogenes* by a log. For the expected low level of contamination with *L. monocytogenes* in foods, this combination treatment may be effective enough to eliminate the pathogen and would add very little extra cost for the processor.

The different physical components of this cheese, such as pH, fat content, and protein content were affected by the processing and formulation treatments used. After production of a soft cheese, processors may manipulate these characteristics through processing changes and addition of extra ingredients to obtain a standard product. These practices are common in the dairy industry. A mixing step is required before packaging to obtain a uniform consistency, so additives could be added at this point. The colorimetric results show that the treatments did not significantly affect the color of this cheese in lightness, redness, or yellowness.

Continued research to discover ways to eliminate *L. monocytogenes* in soft cheeses is very important. The CDC and FDA's goal of reducing the number of food borne listeriosis by 50% by 2010 will not be met without continued research. There are questions from this study that could be answered with further research. The experiment could be repeated with a lower level of contamination that in the cheese, reflective of actual expected contamination from the processing environment, to find the effectiveness of these treatments against a lower amount of *L. monocytogenes*.

There are many other processing and formulation methods for soft cheeses that could be combined with treatments from this study to try to eliminate higher numbers of

L. monocytogenes. Different levels of starter culture could be tested to find which level would offer the greatest destruction of *L. monocytogenes*. An additional treatment involving the use of lactic acid added directly to the milk to raise the acidity of cheese could be added to the study. Utilization of natural antimicrobial spice extracts could be tested to determine if they could offer a greater reduction of *L. monocytogenes*. Any of these treatments could easily be combined with the treatments used in this study. Sensory studies could be conducted to find whether these treatments change the sensory characteristics of the cheese.

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TABLE 1. *Growth and survival of Listeria monocytogenes during production of fromage blanc[1] cheese made using different processing and formulation methods. (N=2)*

Treatment	Population Log (CFU/g)	Population Log (CFU/g) After 24h[6]	Population Log (CFU/g) After 48h[7]
Control[2]	6.4A	-	-
Additional Starter Culture[3] (AS)	6.2AB	-	-
Higher Temperature Draining[4] (HTD)	6.3AB	-	-
AS + HTD	5.5BC	-	-
250ppm Nisin[5] (N)	-	4.1C	4.0C
N + AS	-	5.3C	5.2C
N + HTD	-	4.4C	4.3C
N + AS + HTD	-	4.6C	4.3C

[1] Fromage blanc: Soft cow's milk (pasteurized) cheese

[2] Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*

[3] Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk

[4] Higher temperature draining: drained at 45°C instead of 22°C

[5] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)

[6] After 24h: Cheese sampled 24h after nisin was added and mixed

[7] After 48h: Cheese sampled 48h after nisin was added and mixed

TABLE 2. . *Growth and survival of Listeria monocytogenes during production of fromage blanc[1] cheese made with differing levels of the anti-listerial bacteriocin nisin[2] with comparison to control[3] treatments. (N=2)*

Treatment	Mean Log (CFU/g) After 24h	Mean Log (CFU/g) After 48h
Control	6.4	-
125ppm Nisin[4]	4.3	4.2
250ppm Nisin[5]	4.1	4.0
400ppm Nisin[6]	4.1	3.6

[1] Fromage blanc: Soft cow's milk (pasteurized) cheese
[2] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)
[3] Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*
[4] 125ppm Nisin: Danisco Nisaplin (2.5g added to 500g cheese to obtain a concentration of 125ppm)
[5] 250ppm Nisin: Danisco Nisaplin (5g added to 500g cheese to obtain a concentration of 250ppm)
[6] 400ppm Nisin: Danisco Nisaplin (8g added to 500g cheese to obtain a concentration of 400ppm)

TABLE 3. *Growth and survival of Listeria monocytogenes during production of fromage blanc[1] cheese made using the anti-listerial bacteriocin nisin and other processing and formulation methods. (N=2)*

Treatment	Population Log After 24h[5]	Population Log After 48h[6]
250ppm nisin[2] (N)	4.1C	4.0C
250ppm N + additional starter culture[3] (AS)	5.3A	5.2A
250ppm N + higher temperature draining[4] (HTD)	4.4B	4.3BC
250ppm N + AS + HTD	4.6B	4.3BC

[1] Fromage blanc: Soft cow's milk (pasteurized) cheese
[2] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)
[3] Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk
[4] Higher temperature draining: drained at 45°C instead of 22°C
[5] After 24h: Cheese sampled 24h after nisin was added and mixed
[6] After 48h: Cheese sampled 48h after nisin was added and mixed

TABLE 4. *Mean diameter measurements (mm) of inhibition zones of Lactobacillus sake incorporated into MRS agar[1] by bacteriocin nisin[2] in control samples and in samples of fromage blanc[3], a soft cheese. (N=2)*

Treatment	Mean Diameter of Inhibition Measured in Control Samples (mm)	Mean Diameter of Inhibition Measured in Cheese Samples (mm)
125ppm Nisin[5]	14.0	7.2
250ppm Nisin[6]	14.9	9.2
400ppm Nisin[7]	15.3	9.8

[1] MRS agar: Oxoid CM0361 M.R.S. Agar (de Man, Rogosa, Sharpe)
[2] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive
[3] Fromage blanc: Soft cow's milk (pasteurized) cheese
[4] Nisin Standard: 0.1g Danisco Nisaplin in 10ml 0.02N HCl 0.75% NaCl, pH to 5.3 before steam sterilizing
[5] 125ppm Nisin: Danisco Nisaplin (2.5g added to 500g cheese to obtain a concentration of 125ppm)
[6] 250ppm Nisin: Danisco Nisaplin (5g added to 500g cheese to obtain a concentration of 250ppm)
[7] 400ppm Nisin: Danisco Nisaplin (8g added to 500g cheese to obtain a concentration of 400ppm)

TABLE 5. *Mean values of analytical tests, including pH, fat content, and protein content, of fromage blanc*[1] *cheese made using different processing and formulation methods. (N=2)*

Treatment	pH	Fat Content (percent)	Protein Content (percent)
Control[2]	4.25 C	8.98 CD	8.57 AB
Additional starter culture[3] (AS)	4.28 BC	9.23 BCD	7.99 AB
Higher temperature draining[4] (HTD)	4.24 C	10.18 AB	8.37 AB
AS + HTD	4.22 C	10.68 A	8.35 AB
250ppm nisin[5]	4.41 A	8.59 D	7.21 B
250ppm nisin + AS	4.43 A	8.48 D	8.08 AB
250ppm nisin + HTD	4.37 AB	9.39 BCD	8.42 AB
250ppm nisin + AS + HTD	4.35 AB	9.85 ABC	8.87 A

[1] Fromage blanc: Soft cow's milk (pasteurized) cheese

[2] Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*

[3] Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk

[4] Higher temperature draining: drained at 45°C instead of 22°C

[5] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)

TABLE 6. *Mean colorimeter values, including lightness[1] (L), redness[2] (a), and yellowness[3] (b) of fromage blanc[4] cheese made using different processing and formulation methods. (N=2)*

Treatment	L	a	b
Control[5]	90.59	-1.84	7.64
Additional starter culture[6] (AS)	90.39	-1.78	7.33
Higher temperature draining[7] (HTD)	90.29	-1.74	7.62
AS + HTD	89.57	-1.75	7.64
250ppm nisin[8]	89.39	-1.62	7.42
250ppm nisin + AS	89.21	-1.68	7.30
250ppm nisin + HTD	89.21	-1.61	7.04
250ppm nisin + AS + HTD	88.94	-1.63	7.33

[1] Lightness: Colorimetric measurement in which values approaching 100 indicates very pale, light color

[2] Redness: Colorimetric measurement in which negative values indicate that the color is toward the green direction and positive values indicate toward the red direction on a chromaticity diagram

[3] Yellowness: Colorimetric measurement in which negative values indicate that the color is toward the blue direction and positive values indicate toward the yellow direction on a chromaticity diagram

[4] Fromage blanc: Soft cow's milk (pasteurized) cheese

[5] Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*

[6] Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk

[7] Higher temperature draining: drained at 45^{o}C instead of 22^{o}C

[8] Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)

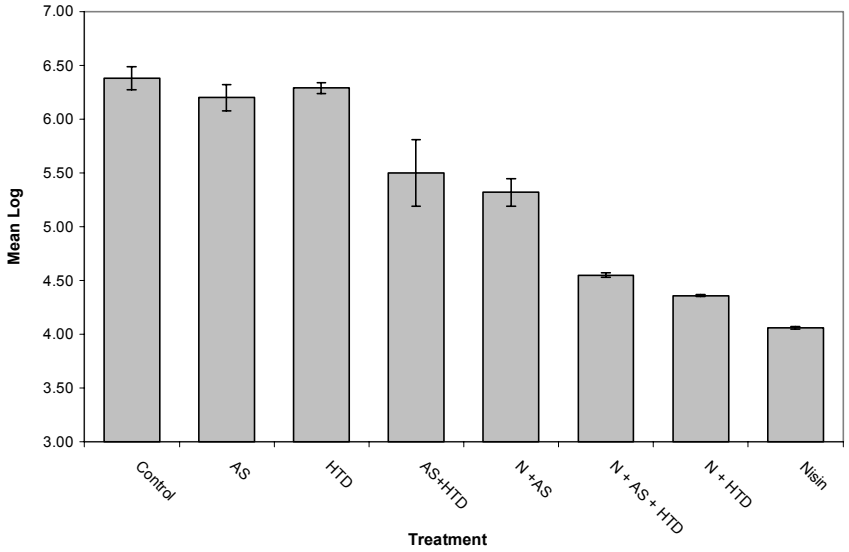

FIGURE 1. *Growth and survival of Listeria monocytogenes during production of fromage blanc cheese made using different processing and formulation methods. (N=2)*

1. Fromage blanc: Soft cow's milk (pasteurized cheese
2. Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*
3. Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk
4. *Higher temperature draining: drained at 45C instead of 22C*
5. *Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)*

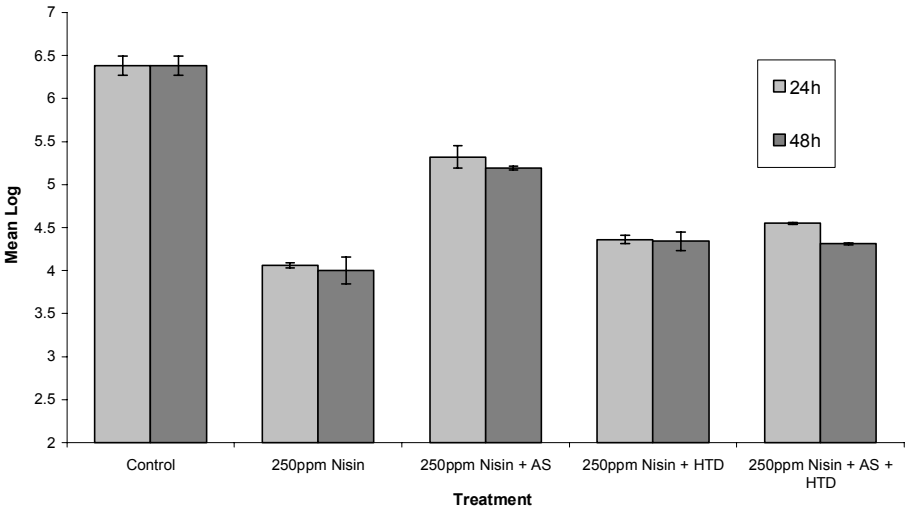

FIGURE 2. *Growth and survival of Listeria monocytogenes during production of fromage blanc cheese made using the anti-listerial bacteriocin nisin and other processing and formulation methods. (N=2)*

1. Fromage blanc: Soft cow's milk (pasteurized cheese
2. Control: Made using Danisco CHOOZIT MM 101 LYO 50 DCU Cheese Cultures (1.0g into 1 gallon milk), contains Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, and Lactococcus lactis subsp. lactis biovar diacetylactis
3.Additional starter culture: 25% (1.25g total) additional starter culture into 1 gallon milk
4.Higher temperature draining: drained at 45C instead of 22C
5.250ppm Nisin: Danisco Nisaplin, Natural Antimicrobial, powdered commercial additive (5g added to 500g cheese to obtain a concentration of 250ppm)

VITA

Emily Mathusa was born in Fairfax, VA and raised in Stafford, VA. She attended Brooke Point High School for two years, and then Colonial Forge High School where she was part of its first graduating class. After graduating high school, she attended George Mason University in Fairfax, VA. In 2005, she graduated with honors, earning a Bachelor of Science in biology with a concentration in microbiology. While attending George Mason University, she participated in the university's chapter of the American Society for Microbiology, serving as president for two years. She also held an undergraduate research assistantship with Dr. Paulette Royt, in which she studied the chemical properties of an iron chelator produced by *Pseudomonas ariginosa* and also challenged four bacterial species with extracts from a plant with suspected antimicrobial qualities. She also held a student staff position as a laboratory preparation assistant in the Biology Department for over two years. Upon graduating, she was awarded with the student faculty member of the year award for the fall 2004 through spring 2005 academic year. She also volunteered in many youth science related activities during her undergraduate studies.

In 2005, Emily entered the Department of Food Science and Technology at Virginia Tech where she is currently a Masters Candidate under Dr. Susan Sumner. While in graduate school, she participated in the department's Food Science Club and Food Product Development Team. She served as a delegate in the Graduate Student Assembly, representing her department. She was also a student member of the International Association of Food Protection and the Institute of Food Technologists.

Emily participated as a volunteer for the Virginia Tech Women's Center, mainly in the youth girls program, AWARE.