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**EVALUATION AND EXPLORATION OF MICROBIAL QUALITY AND SAFETY
ASPECTS OF UNPASTEURIZED RETAIL MAINE DAIRY PRODUCTS**

By

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A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy of Science

(in Food Science and Human Nutrition/Food Microbiology)

The Graduate School

The University of Maine

May 2020

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Dissertation Advisor: Dr. Jennifer J. Perry

An Abstract of the Dissertation Presented
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May 2020

The Dietary Guidelines for Americans recommend dairy products as part of a healthy diet and these products are significant contributors of important nutrients. In the U.S., and Maine specifically, demand for locally-produced, minimally processed foods, including unpasteurized dairy, has increased during the past several decades.

An analysis of retrospective microbial testing data for unpasteurized retail dairy products revealed increasing microbial quality from 1998-2016, despite a five-fold increase in sample numbers during this time. Higher percentages of samples were non-compliant with microbial quality standards during the summer months compared to winter. High coliform counts were the leading cause of non-compliant samples in milks and other dairy-based products.

The second objective of this research was to investigate the effects of cheesemaking and aging temperature on levels of inoculated foodborne pathogens. The cheesemaking process did not affect the populations of either pathogen of interest. During aging, lower temperatures (4°C and 10°C) significantly decreased population of Shiga-toxigenic *E. coli*, whereas only 4°C significantly reduced *L. monocytogenes*. Aging at 4°C was also effective in maintaining the population of the

starter culture. The highest aging temperature (22°C) caused a significant increase of both pathogens as well as a significant increase in pH levels and softening of the cheese texture.

In tests of unpasteurized retail dairy products, the highest rates of presumptive positive results for *Listeria* spp. were recorded in July and August. The prevalence of presumptive positive *Listeria* spp. was higher in mold-ripened compared to soft cheeses. There was a significant positive correlation between non-compliant levels of coliforms and presence of presumptive positive *Listeria* spp. in the samples tested.

The results of this research suggest that the overall quality of the expanding unpasteurized dairy sector in Maine has remained constant or improved in the past two decades. Small-scale cheese operations participating in this market should use refrigeration temperatures for aging of cheese when possible, which will provide the best control against foodborne pathogens. Finally, more targeted testing techniques are suggested to detect the potential sources of coliforms and to ensure the safety of unpasteurized dairy commodities.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF TABLES	iv
LIST OF FIGURES	v
INTRODUCTION	1
CHAPTER 1 LITERATURE REVIEW	3
1.1 Milk Biosynthesis and Composition	3
1.2 Natural Microflora of Milk	5
1.2.1 Psychrotrophic Bacteria	5
1.2.2 Lactic Acid Bacteria	6
1.2.3 Coliform Bacteria	7
1.3 Milk Quality	7
1.3.1 Standard Plate Count	8
1.3.2 Coliform Count	8
1.4 Hygienic Practices During Milking	9
1.5 Milk-borne Pathogens	10
1.5.1 <i>Listeria monocytogenes</i>	10
1.5.1.1 Characterization	10
1.5.1.2 Listeriosis	11

1.5.1.3	Prevalence of <i>L. monocytogenes</i> in Food Processing.....	11
1.5.2	<i>Escherichia coli</i>	12
1.5.2.1	Characterization.....	12
1.5.2.2	Hemorrhagic Colitis.....	13
1.5.2.3	Prevalence of <i>E. coli</i> in Food Processing.....	13
1.6	Public Health Concerns.....	14
1.6.1	Foodborne Disease Outbreaks in the United States.....	16
1.6.1.1	Foodborne Disease Outbreaks Associated with Pasteurized and Unpasteurized Dairy Products.....	17
1.7	Regulations of Selling Unpasteurized Milk and Milk Products.....	19
1.8	Maine Dairy Industry.....	19
1.9	Cheese Classification.....	20
1.9.1	Hard Cheeses.....	20
1.9.2	Ripened Cheeses.....	21
1.9.3	Soft Cheeses.....	21
1.10	Cheese Processing.....	22
1.10.1	Ripening.....	23
1.10.2	Coagulation.....	24
1.10.2.1	Acid Coagulation.....	24
1.10.2.2	Acid/Heat Coagulation.....	25

1.10.2.3 Rennet Coagulation.....	26
1.10.3 Cutting.....	27
1.10.4 Heating the Curds.....	27
1.10.5 Releasing the Whey.....	27
1.10.6 Molding and Pressing.....	28
1.10.7 Aging.....	29
1.10.7.1 Proteolysis.....	30
1.10.7.2 Lipolysis.....	31
CHAPTER 2 RETROSPECTIVE ANALYSIS OF MICROBIAL QUALITY OF UNPASTEURIZED RETAIL MILK AND FLUID DAIRY PRODUCTS IN MAINE BETWEEN 1998 AND 2016.....	32
2.1 INTRODUCTION.....	32
2.2 MATERIALS AND METHODS.....	34
2.2.1 Data.....	34
2.2.2 Descriptive Analyses.....	35
2.2.3 Statistical Analyses.....	35
2.3 RESULTS.....	35
2.3.1 Bacterial Populations of Unpasteurized Retail Goat and Cow Milk Samples.....	35
2.3.2 The Seasonal Effects on the Bacterial Populations of Unpasteurized Fluid Retail Cow and Goat Milk Samples.....	38

2.3.3	The Microbial Quality of other Unpasteurized Fluid Dairy Products	41
2.4	DISCUSSION	42
2.5	CONCLUSIONS	46
CHAPTER 3 THE DYNAMIC BEHAVIOR AND SURVIVAL OF <i>LISTERIA</i>		
<i>MONOCYTOGENES</i> AND SHIGATOXIGENIC <i>ESCHERICHIA COLI</i> DURING THE		
AGING OF FARMSTEAD-STYLE CHEESE		
		48
3.1	INTRODUCTION.....	48
3.2	MATERIALS AND METHODS	51
3.2.1	Bacterial Strains	51
3.2.2	Inoculum Preparation	51
3.2.3	Starter Culture	51
3.2.4	Milk Collection.....	52
3.2.5	Cheese Manufacturing.....	52
3.2.6	Vacuum Packaging.....	53
3.2.7	Cheese Samples and Aging Conditions.....	53
3.2.8	Bacterial Enumeration and Detection.....	54
3.2.9	Enumeration of Fungi.....	55
3.2.10	Measuring the pH and Water Activity Levels.....	55
3.2.11	Texture Profile Analysis (TPA)	55
3.2.12	Statistical Analysis	56

3.3	RESULTS.....	56
3.3.1	Milk	56
3.3.2	The Behavior of Pathogens during Farmstead-style Cheese Manufacturing	56
3.3.3	The Survival of Pathogens during the Aging of Unpasteurized Cheese	57
3.3.4	The Effects of Temperature on Lactic Acid Bacteria (LAB) Populations During the Aging Process of Unpasteurized Cheese	60
3.3.5	The Effects of Aging Temperatures on the pH Levels of Unpasteurized Cheese during Aging.....	62
3.3.6	The Effects of Aging Temperature on the Water Activity Levels and Texture of Unpasteurized Cheese during Aging	63
3.3.7	The Effects of Temperature on Fungal Counts in Unpasteurized Cheese After Aging	64
3.4	DISCUSSION	65
3.5	CONCLUSIONS	73
3.6	LIMITATIONS	74
CHAPTER 4 DETECTION OF <i>LISTERIA</i> SPP. IN UNPASTEURIZED RETAIL DIARY PRODUCTS IN MAINE		
		75
4.1	INTRODUCTION.....	75
4.2	MATERIALS AND METHODS	77
4.2.1	Samples	77
4.2.2	Microbiological Analysis	78

4.2.3	Statistical Analysis	79
4.3	RESULTS.....	79
4.3.1	The Correlation between <i>Listeria</i> spp. and Coliforms in Unpasteurized Retail Dairy Products.....	79
4.3.2	The Prevalence of <i>Listeria</i> spp. and Coliforms in Different Types of Unpasteurized Retail Cheese.....	80
4.3.3	The Effect of Months on the Prevalence of <i>Listeria</i> spp. and Coliforms in Unpasteurized Retail Dairy Samples.....	81
4.4	DISCUSSION	82
4.5	CONCLUSION	87
4.6	LIMITATIONS	88
	OVERALL CONCLUSIONS AND RECOMMENDATIONS.....	89
	REFERENCES	91
	BIBLIOGRAPHY OF THE AUTHOR	117

LIST OF TABLES

Table 1-1 Common Milk Pasteurization Processes.....	15
Table 1-2 Estimated Annual Number of Foodborne Illnesses in the United States Caused by Prevalent Pathogens	16
Table 1-3 Recent Multi-state Recalls of Cheese Testing Positive for <i>Listeria monocytogenes</i> in the United States.....	18
Table 1-4 Cheese Texture Classification Based on Firmness and Ripening Characteristics	20
Table 2-1 The Percentage of Compliant and Non-Compliant Bacterial Populations in Unpasteurized Retail Milk Samples.....	38
Table 3-1 Unpasteurized Cheese Texture Analysis.....	64
Table 4-1 Pearson Correlation Between Coliforms and <i>Listeria</i> spp. in Unpasteurized Maine Dairy Products.....	80
Table 4- 2 Prevalence of <i>Listeria</i> spp. in Different Types of Unpasteurized Retail Cheese.....	80
Table 4- 3 Prevalence of <i>Listeria</i> spp. and Coliforms during the Study in Unpasteurized Maine Dairy Products.....	81

LIST OF FIGURES

Figure 1-1 The Proposed Structure of Casein Micelles.....	5
Figure 1-2 The Cheesemaking Process.....	24
Figure 2-1 The Mean Bacterial Populations Values between 1998 and 2016 in Unpasteurized Retail (A): Cow’s Milk and (B): Goat’s Milk.....	37
Figure 2-2 The Season Effects on Bacterial Populations in Unpasteurized Retail Cow’s Milk (2A and 2B) and Goat’s Milk (2C and 2D)	40
Figure 2-3 The Effects of Different Types of Unpasteurized Retail Cow’s Milk Products on Bacterial Populations.....	41
Figure 3-1 Behavior of <i>E. coli</i> and <i>L. monocytogenes</i> during Cheese Manufacturing.....	57
Figure 3-2 The Effect of Temperature on the Survival of <i>E. coli</i> (A) and <i>L. monocytogenes</i> (B) during Cheese Manufacturing.....	59
Figure 3-3 The Effect of Temperature on Lactic Acid Bacteria (LAB) counts (A) Control Cheese and (B) Inoculated Cheese samples During the Cheese Aging Process.....	61
Figure 3-4 The Effects of Temperatures on pH values of Cheese During the Cheese Aging Process.....	62
Figure 3-5 The Effect of Temperature on the Water Activity Levels During the Aging Process.....	63
Figure 3-6 The Effect of Temperature on the Fungi Population at the End of the Aging Process.....	65

Figure 4-1 The Trend of Maine’s Temperatures Across Study Duration.....82

INTRODUCTION

Milk and dairy products are an important component of the typical American diet. In addition to the more mainstream pasteurized dairy retail space, the market for unpasteurized dairy continues to grow in popularity throughout the U.S. Despite its increasing demand, the sale and distribution of these products is highly contentious, as a result of the potential consumer safety hazards associated with these products. Therefore, the sale of unpasteurized dairy is legal within only thirty states, twelve of which, including Maine, permit the sale within retail stores. Maine is one of the largest producers of unpasteurized dairy products, which contributes \$570 million each year to the state's economy¹. High quality milk plays an important role, not only for its consumption, but also for producing other dairy-based products. Different microbial tests have been used to evaluate the quality of these food commodities. Among these tests, standard plate and coliform counts are the most frequent methodologies that are used to assess the overall quality and hygienic conditions during milking, as well as to assess the presence of any fecal contamination^{2,3}. There are several factors which can influence the initial microbial milk populations including milking procedures, milk handling, hygienic practices, and animal health status^{4,5}. Seasonal variations in milk quality, yield, and compositions have also been investigated. Previous studies have shown that higher coliform, standard plate and somatic cell counts were observed in the summer compared to the winter^{6,7}. Additionally, milk production yields and the milk fat and protein content were found to be lower in summer rather than in winter and spring months^{8,9}.

Proper hygienic practices during milking can help to maintain milk quality and minimize the introduction of contaminants, better assuring the safety of the finished product. The popularity and the production of unpasteurized dairy products, namely cheese, have expanded in the U.S. and worldwide; however, safety of unpasteurized dairy products is still questionable. The current FDA

regulations (21 CFR 133) allow the interstate sale of cheeses made from unpasteurized milk after aging for at least 60 days at temperatures of 35°F (1.67°C) or above to ensure product safety¹⁰. Several studies have found that 60 days of aging was not enough to eliminate *E. coli* O157:H7, or *L. monocytogenes*, from cheeses that were made from either pasteurized or unpasteurized milk¹¹⁻¹⁵.

In addition to sanitation practices, there are several complex considerations which contribute to the overall microbial quality and safety of the finished product. Intrinsic factors include pH and water activity levels, nutrient content, redox potential, and the presence of antimicrobial substances, which are inherent to the product. Beyond these inherent attributes, processing variables present several external factors which can greatly influence microbial safety¹⁶. These include packaging and relative humidity in the processing environment, as well as production steps used by the producer such as aging conditions^{12,17-21}.

To date, there is a shortage in the literature of systematic investigations regarding the quality of unpasteurized retail dairy products; the survival of foodborne pathogens such as non-O157 *E. coli* serotypes and *L. monocytogenes* during the manufacturing of these products within small scale cheese operations, and the correlation between the presence of *Listeria* spp. and coliforms in unpasteurized retail dairy products. Thus, the primary objectives of this research were: (1) to analyze the retrospective microbial quality of unpasteurized retail milk and fluid dairy products in Maine between 1998 and 2016, (2) to investigate the dynamic behavior and survival of *Listeria monocytogenes* and Shigatoxigenic *Escherichia coli* during the aging of farmstead-style cheese, and (3) to screen for the presence of *Listeria* spp. in unpasteurized retail dairy products in Maine.

CHAPTER 1 LITERATURE REVIEW

1.1 Milk Biosynthesis and Composition

Milk is defined as “the colostrum free lacteal secretion, obtained by the complete milking of one or more healthy cows” 21CFR133.110²². The rumen (the first stomach of the ruminant digestive system) allows the cow to synthesize nutrients from plant materials (cellulosic and fibrous) and from simple sources of nitrogen such as urea²³. Milk is synthesized in special cells of the mammary gland and stored in the alveoli. Alveoli are spherical micro-organs consisting of central storages known as lumens. Each lumen is surrounded by a single layer of secretory epithelial cells, which is then connected to the duct system of the udder²⁴.

The major components of cow's milk are water, proteins, fats, lactose, and salts, which constitute of approximately 86.6%, 3.6%, 4.1%, 5.0% and 0.7% of milk, respectively²⁵. These components are synthesized from various precursors from the blood stream, including amino acids, blood sugar, and fatty acids, which are converted (metabolized) to proteins, lactose, and fats, respectively, throughout different pathways in the secretory cells²⁶. In milk, water is the predominant liquid phase and is the matrix in which all other solid constituents are dispersed²⁷. Lactose is a polar disaccharide available in milk and comprises approximately 5% of the total milk composition. Cow's milk also has a complex lipid profile. These lipids include triglycerides, diglycerides, monoglycerides, free fatty acids, phospholipids, cholesterol, cholesterol ester, and hydrocarbons, which are approximately 98% of milk fats, collectively²⁸. In addition, the most predominant milk salts are chlorides, citrates, phosphates, carbonates, sulfates and bicarbonates of sodium, calcium, potassium, and magnesium. Other elements such as zinc, lead, copper, iron, iodine, manganese and boron are also found in trace quantities²⁸.

Milk proteins consist of two classes: caseins and whey proteins. Casein, which comprise 80% of total milk proteins are further subdivided into four major molecules: α_{s1} , α_{s2} , β , and κ -casein. These constituents represent 38%, 10%, 34%, and 15% of the casein, respectively²⁹. Due to unique interactions with calcium ions and calcium salts; α_{s1} , α_{s2} , and β are known as calcium-sensitive caseins, whereas κ -caseins are considered calcium-insensitive²⁴. Casein proteins are tangled in a spherical mass of thousands of individual casein molecules that are linked together in part by calcium phosphate nanocrystals through ionic linkages with phosphate serine residues on adjacent casein molecules, known as casein micelles^{27,347} (Figure 1- 1). There are three primary characteristics of these micelles; first is polarity, which is due to the high κ -casein concentration on the micelles' surface. This polar surface enables the micelle to interact with water molecules and remain dispersed in the milk water phase²⁷. Second is buffering capability to absorb hydrogen ions. This buffering action is due to the high calcium phosphate content within the casein micelles, which enables the micelles to absorb the ions and the micellar calcium phosphate is then converted to a more soluble form and is released from the micelles into the water phase. Finally, casein micelles possess strong water-binding and water-holding capacities. The water-binding capacity of the micelle within a cheese matrix, the proteins within the micelle, bind with available water making it less accessible for microorganisms to utilize. Additionally, the reduced water accessibility within this type of medium helps to reduce enzymatic reactions.

On the other hand, whey proteins represent 20% of the total milk proteins and consist of serum albumin, immunoglobulins, alpha-lactalbumin, and beta-lactalbumin. These proteins are available as monomers or dimers that are folded into compact globular, three-dimensional structures³⁰. Normally, whey proteins are heat-susceptible and start to denature and unbind through

exposure of their nonpolar hydrophobic sites to temperatures above 79°C. Similarly, denaturation can occur when the pH of milk is reduced to a level of 6.0 or lower³⁰.

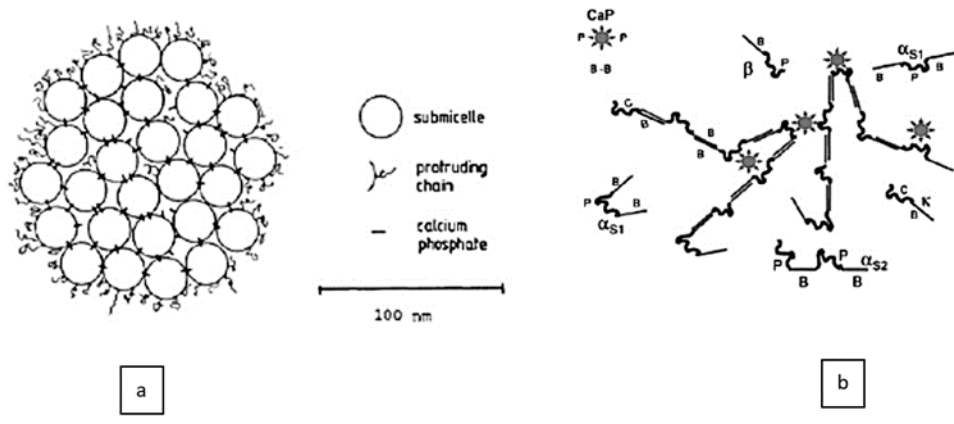


Figure 1- 1 The Proposed Structure of Casein Micelles

1.2 Natural Microflora of Milk

Generally, fresh milk from a healthy cow is free of pathogens; however, shortly after milking, milk becomes colonized by microbes from several sources, including the teat apex, milking equipment, air, soil, grass and water³¹⁻³³. The average level of aerobic microorganisms in raw milk is between 10^3 - 10^4 CFU/ml³⁴. The most common microorganisms that exist in milk are lactic acid bacteria (LAB), psychrotrophs, yeast and molds³⁵. Several factors, such as the environment where animals are kept, feeding location (outdoors versus indoors), and the stage of lactation have been found to affect the diversity of milk microflora³⁵⁻³⁷.

1.2.1 Psychrotrophic Bacteria

Psychrotrophic bacteria are microorganisms that are able to reproduce at low temperatures (3-7°C)³⁸ with optimal metabolic activity temperatures that range between 20-30°C³⁹. The most common genus of Gram-negative psychrotrophs is *Pseudomonas*, which accounts for approximately 65-70% of the psychrotrophs that are isolated from raw milk⁴⁰. Other psychrotrophs

isolated from raw milk include *Acinetobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, and *Flavobacterium*³⁹. The predominant Gram-positive psychrotrophs in milk are *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, and *Staphylococcus*³⁹. Milking equipment contaminated by soil, water, and vegetation are the primary sources of psychrotrophs in milk. Generally, this group of bacteria comprises of less than 10% of the microflora of freshly drawn milk if proper hygienic practices have been followed throughout milking. Otherwise, psychrotrophs in milk may comprise more than 70% of the total bacterial load⁴¹. Some psychrotrophs, such as *Pseudomonas* and *Bacillus*, can produce extracellular heat resistant lipases, proteases and phospholipases^{42,43}, which can have a negative effect on the product shelf life⁴⁴. Psychrotrophs are susceptible to pasteurization; thus, their presence in pasteurized milk indicates either post-process contamination or inadequate pasteurization^{38,45}. However, psychrotrophic spore-forming bacteria, such as *Bacillus* and *Clostridium*, are another cause of spoilage. These spore-forming bacteria are capable of surviving pasteurization temperatures and the spore form and can germinate under normal storage conditions⁴⁶. Generally, silage, soil, pasture, and bedding materials are considered the main sources of spores in the dairy farm environment⁴⁶⁻⁴⁹.

1.2.2 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are naturally present as indigenous microflora in raw milk⁵⁰. LAB genera are predominantly Gram-positive, cocci and bacilli in shape, strictly fermentative, acid tolerant, and are naturally present in the bovine mammary gland⁵¹. The most common genera of LAB found in milk are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus*. Cow's milk has a significant LAB population ranging between 10¹-10⁴ CFU/ml⁵². LAB have been used as starter cultures in the production of fermented foods and beverages due to

a long and safe history of application and consumption and are considered Generally Recognized As Safe (GRAS) by the FDA⁵³. LAB have a significant role in cheesemaking due to their use for rapid acidification by converting of milk sugar (lactose) to lactic acid, resulting in the precipitation of milk proteins (casein)⁵⁴. Besides this characteristic, LAB also produces organic acids, antimicrobial agents, and enzymes which are critical to both ensuring finished product safety and maintaining cheese quality⁵⁵.

1.2.3 Coliform Bacteria

Coliform bacteria are Gram-negative rods and ferment lactose with the production of acid and gas. They are aerobic or facultative anaerobic bacteria. Coliforms include the following genera: *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*⁵⁶. Typically, sources of coliforms are feces, bedding materials, soil, and water. Thus, their presence in the farm environment is very likely, however, rigorous sanitation programs will prevent coliform introduction into the finished product². Coliforms are susceptible to pasteurization temperatures; therefore, their presence in pasteurized milk indicates either post-pasteurization contamination or inadequate processing⁵⁶.

1.3 Milk Quality

Raw milk quality is evaluated on both the macronutrient composition (fat and protein content) and the hygiene status (low bacterial and somatic cell counts)⁵⁷. High quality milk is important for the production of high-quality dairy products. There are numerous microbiological tests used to evaluate milk and milk product quality, including the standard plate count (SPC), coliform count (CC), somatic cell count (SCC), plate loop count (PLC), the preliminary incubation count (PIC), and the laboratory pasteurization count (LPC)⁵⁸. In the U.S., the present regulatory limit for high quality “Grade A” for bacterial counts are less than 100,000 CFU/ml and 750,000

cells/ml for SPC and SCC, respectively, as well as the “absence of foodborne pathogens”⁵⁸. In retrospect, both the SPC and CC have been used predominantly to assess the quality of dairy products³⁸.

1.3.1 Standard Plate Count

The standard plate count is a procedure that measures the total bacterial count in a milk sample, although it is known to underestimate certain populations. In this procedure, serial dilution and plating on a nonselective agar are used to determine the number of colony forming units (CFU) in one ml after incubating plates at 35°C for 48 hours⁵⁹. The Petrifilm™ Aerobic Count (PAC) is a version of this methodology which uses a dehydrated, commercially available medium⁶⁰. This method is more widely utilized in industry and is the current method of analysis at the Milk Quality Laboratory at the Maine Department of Agriculture, Conservation and Forestry in Augusta, Maine⁶¹. Under ideal circumstances, the SPC level in the milk from clean and healthy cows is generally less than 1,000 CFU/ml; however, when sanitation and cooling practices are adequate, an SPC level of ~5,000 CFU/ml is common for most farms⁶⁰.

1.3.2 Coliform Count

Coliform testing is used as indicator of fecal contamination. As required by the PMO, “Grade A” pasteurized milk must contain coliform counts (CC) of less than or equal to 10 CFU/ml⁵⁸. Coliforms typically associated with fecal origins include, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter* spp.⁶². These bacteria are natural inhabitants of the gastrointestinal tract of warm-blooded animals, therefore, their presence in fluid milk and dairy products are not surprising^{63,64}. Under proper hygienic milking practices, the typical level of coliforms in raw milk samples should be between 50-100 CFU/ml, however, 25 CFU/ml is

achievable in most dairy operations⁶⁰. Although there are no legal coliform count limits for unpasteurized milk samples, as outlined under the PMO, these limits are set within each state that allows the sale of unpasteurized milk^{58,65}. Coliform testing is performed by plating milk samples on violet red bile agar (VRBA), however, the Petrifilm™ coliform count is an accepted alternative method which is currently used by the Milk Quality Laboratory to evaluate the quality of unpasteurized retail fluid milk and milk products in Maine markets⁶¹.

1.4 Hygienic Practices During Milking

Milk is a highly nutritious food product that serves as an optimal medium for microbial growth. Microorganisms can enter the milk from numerous sources, including the udder surface, milking equipment and tools, as well as the dairy farm environment⁶⁶⁻⁶⁸. Consequently, the initial total bacterial count of milk can vary depending on several factors, such as hygienic practices, milking procedures, milk handling, and animal health status⁶⁹. The reduction of bacteria on teat skin decreases the contamination of milk, and thus enhances milk quality⁷⁰. Both farm conditions and management practices can either reduce or improve the efficacy of pre-milking teat sanitation methods⁷¹. Sanitation of the teat end is a crucial step in reducing the bacterial count before the milking process, and it helps in controlling mastitis and udder health⁶⁹. The most common contagious pathogens that can cause mastitis are *Staphylococcus aureus* and *Streptococcus agalactiae*⁷². Higher somatic cell counts (SCC) in milk samples can be an indication of mastitis, which is defined as an inflammatory disease that infects the mammary tissues of cattle or other lactating mammals⁷². Milk that has a high SCC (> 750,000 cells/ml) is not permitted for sale^{73,74}.

Pre and post-milking teat dipping have been found to be the most effective procedures in controlling mastitis pathogens compared to just a post-milking teat dipping procedure⁷⁵. Common commercial sanitizers used for teat dipping include Clean™ 1.75% iodine, Tri-Fender™ 1%

iodine and Teat Cleaner NI01™ lactic acid/formic acid, which are all approved by the FDA⁷⁶. For Maine and dairy farms nationwide, the pre-dipping procedure is recommended by the National Mastitis Council (NMC). Several studies have found that pre- and post-milking teat dipping procedures were effective in reducing mastitis-associated bacteria and the total bacterial count on the teat skin surface⁷⁷⁻⁸². Proper milking practices have significant effects on the milk quality and also on milk safety by minimizing the chances of contamination with foodborne pathogens.

1.5 Milk-borne Pathogens

The neutral pH and nutritional value of milk make it an optimal medium for unwanted microbial growth⁸³. In the past, *Mycobacterium bovis*, *Brucella abortus*, and *Coxiella burnetii* were the major pathogens of concern in milk. These pathogens have been completely eradicated from dairy herds in several countries⁸⁴; however, these pathogens continue to be endemic in many regions⁸⁵ and more specifically, *M. bovis* has re-emerged in some places where it had formerly been excluded⁸⁶. Currently the most common foodborne pathogens of concern associated with raw milk are *S. aureus*, *Salmonella* spp., Shiga-toxigenic *Escherichia coli* (STEC), *L. monocytogenes*, and *Campylobacter* spp.⁶⁶. Several studies have documented that the most prevalent foodborne pathogens in bulk tank milk (BTM) samples are *Salmonella* spp., STEC, *L. monocytogenes*, and *Campylobacter jejuni*⁸⁷⁻⁸⁹.

1.5.1 Listeria monocytogenes

1.5.1.1 Characterization

Listeria monocytogenes is an intracellular, Gram-positive rod approximately 1-2 µm in length and 0.5 µm wide⁹⁰. It is a non-spore-forming, aerobic or facultative anaerobe⁹¹. *L. monocytogenes* can reproduce in a wide range of environmental conditions, including pH levels of

4.5-9.0, high salt media (10-20% W/V of NaCl), water activity as low as 0.92 and temperatures between 2-45°C^{92,93}. The motility characteristics of this pathogen is characterized by incubating the cultures at room temperature; however, at 37°C the organism can be non-motile⁹⁴.

1.5.1.2 Listeriosis

Listeriosis is a disease caused by *L. monocytogenes* and is estimated to affect 1,600 people each year in the U.S.⁹⁵. The infective dose of this pathogen is undetermined; however, it varies based on the pathogen strain, the food product in which it is present, and the health of the human host⁹⁶. There are two types of listeriosis: the first is invasive, which is more severe and affects the population at the highest risk including pregnant women, the elderly and immunocompromised individuals. This type causes fever, muscle pain, meningitis, and septicemia and has a long incubation period (3-90 days). The second type is non-invasive, which affects mainly otherwise healthy individuals, and the symptoms are fever, headache, and muscle pain with a short incubation period of just a few days⁹⁶. According to epidemiological data, listeriosis has one of the highest hospitalization rates and one of the highest cases of fatality rates among foodborne diseases in the U.S.^{97,98}.

1.5.1.3 Prevalence of *L. monocytogenes* in Food Processing

Listeria monocytogenes is present in soil, decayed vegetation, silage, water, and is considered a natural environmental pathogen⁹⁹⁻¹⁰². *L. monocytogenes* is the foodborne pathogen of most concern in food processing facilities that produce ready-to-eat (RTE) foods¹⁰³. This bacterium has been isolated from different variety of foods, for example, raw and pasteurized milk, cheese (especially soft-ripened), ice cream, fermented raw meat and cooked sausages, raw and cooked poultry, raw meats, raw and cooked seafood, and raw and frozen vegetables¹⁰⁴. Manure

and improperly fermented silage are also considered possible sources of *L. monocytogenes* in the dairy farm environment¹⁰⁵.

L. monocytogenes can survive for long periods in undesirable environmental conditions. In addition to survival and growth at refrigeration temperatures, this pathogen tolerates high salt and acid conditions¹⁰⁶. Furthermore, it is capable of surviving frozen storage conditions for extended periods¹⁰⁷ and is more heat resistant in comparison to other non-spore forming pathogens, although it can be killed by cooking and pasteurization temperatures¹⁰⁸. Despite best efforts in eliminating *Listeria* spp. from food processing environments, *Listeria* can potentially be reintroduced into the facility¹⁰⁹. *L. monocytogenes* can establish and form biofilms in difficult to clean areas within processing environments¹¹⁰, and thus, persist for a prolonged period of time. The control of this pathogen is required at all food processing steps and strict sanitation procedures are needed to prevent contamination with this pathogen, especially since the U.S. has a zero-tolerance of final food products.

1.5.2 Escherichia coli

1.5.2.1 Characterization

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobic, and many strains harmlessly colonize the gastrointestinal intestinal tract of healthy humans and animals¹¹¹. *E. coli* can grow within a wide range of environmental conditions including temperatures between 7 and 50°C with an optimum temperature of 37°C, can survive at low pH levels and minimum water activity level of 0.95¹¹². The serological characteristics of *E. coli* are determined in great part by the O antigens, which are lipopolypeptides on the bacterial surface and their polysaccharide moiety has an important role in serological specificity and the H (flagellar) antigens¹¹². There are six major groups (pathotypes) of pathogenic *E. coli* including enteropathogenic *E. coli* (EPEC), Shiga toxin-

producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC)¹¹¹.

1.5.2.2 Hemorrhagic Colitis

STEC has caused illnesses associated with the consumption of different types of foods, including unpasteurized dairy products¹¹³. STEC strains are characterized to cause hemorrhagic colitis (HC) with bloody diarrhea, which may progress to hemolytic uremic syndrome (HUS). HUS is defined as the most common cause of acute renal failure in children¹¹⁴. The estimated infective dose of STEC is between 10 to 100 cells and the onset of the disease begins three to four days after ingesting contaminated food¹¹⁵.

1.5.2.3 Prevalence of *E. coli* in Food Processing

Natural sources of *E. coli* include soil and the feces of humans and animals; thus, the presence of this bacterium is considered an indication of fecal contamination of food and water¹¹⁶. Both pathogenic and non-pathogenic strains of *E. coli* are present in the dairy farm environment since dairy cattle are a reservoir for them^{117,118}. Currently, O157:H7 accounts for approximately 75% of the STEC infections worldwide; however, non-O157 STEC serotypes (including O111, O26, O121, O103, O145, and O45) are also considered emerging sources of foodborne illnesses¹¹⁹⁻¹²¹. Prevention and control of foodborne pathogens in different food sectors require various approaches include Good Agriculture Practices (GAP), Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP), and the use of Hazard Analysis Critical Control Point (HACCP) programs¹²².

There are other common safety hazards associated with milk and dairy products, including *Campylobacter*, *Salmonella*, and *S. aureus*⁶⁶. The CDC estimates that *Campylobacter* spp. cause 1.3 million illnesses each year in the U.S. and is responsible for the cause of campylobacteriosis.

Typical symptoms include fever, diarrhea, abdominal cramps, and vomiting. Sometimes the stool may be watery or sticky and may contain blood. Generally, the ingestion of food and water contaminated with animal and/or bird feces is the source of campylobacteriosis¹²³⁻¹²⁵. *Salmonella* is one of the most prevalent foodborne pathogens worldwide¹²⁶ and the CDC estimates *Salmonella* causes about 1.2 million illnesses, 23,000 hospitalizations and 450 deaths in the U.S. every year. *Salmonella* can cause salmonellosis and the symptoms of this disease are vomiting, abdominal cramps, diarrhea, fever and headaches. *S. aureus* can cause staphylococcal (Staph) food poisoning, which is a gastrointestinal illness. This illness is characterized by nausea, abdominal cramping, vomiting, diarrhea, muscle cramping, headaches, and dehydration¹²⁷.

1.6 Public Health Concerns

Milk and milk products possess nutritional benefits; however, unpasteurized milk can harbor pathogenic microorganisms that can cause serious health consequences for humans. The most common foodborne pathogens associated with the consumption of unpasteurized milk and dairy products include *Campylobacter*, *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*^{128,129}. In 1948, Michigan was the first state in the United States to enact the requirement of pasteurization for dairy products¹³⁰. Pasteurization is the process of heating milk to a specific temperature for an established period of time⁵⁸. Pasteurization is aimed to enhance safety and extend the shelf life of the food product. Unlike sterilization, pasteurization is designed to kill all vegetative cells of pathogenic bacteria such as *E. coli* O157:H7, *Salmonella*, *L. monocytogenes*, *M. tuberculosis*, *C. burnetti*, and *Campylobacter*. Nonetheless, thermophilic bacteria and spore-formers (*Bacillus* and *Clostridium*) can survive pasteurization, grow and spoil milk at refrigeration temperatures¹³¹. Pasteurization also inactivates some milk enzymes, such as lipase and alkaline phosphatase. Inactivation of alkaline phosphatase is used as an indicator of effective pasteurization¹³². Failure

of lipase inactivation can cause rancidity development in milk due to the production of free fatty acids, specifically butyric acid. The two most common pasteurization processes are low temperature-long time (LTLT) and high temperature-short time (HTST) pasteurization as seen in (Table 1- 1) LTLT is commonly known as batch or vat pasteurization, which aims to heat milk to 145°F (63°C) for 30 minutes, whereas HTST is known as a continuous process and is achieved by heating the milk to 161°F (72°C) for 15 seconds⁵⁸. The other thermal treatment commonly used in the dairy industry is ultra-high temperature (UHT) pasteurization. This is achieved by heating the milk to 275-284°F (135-140°C) for a few seconds to destroy non-spore forming pathogens.

Table 1- 1 Common Milk Pasteurization Processes⁵⁸

Pasteurization Type	Temperature/Time	Shelf-life of Milk
Batch/Vat Pasteurization (LTLT).	63°C (145°F)/30 minutes	7-10 days at refrigeration temperature.
Continuous Flow (HTST).	72°C (161°F)/15 seconds.	7-10 days at refrigeration temperature.
Ultra-Pasteurization (UP).	135°C (280°F)/2 seconds.	60-90 days at refrigeration.
Ultra-High Temperature (UHT).	135-140°C (275-284°F)/1-2 seconds.	90 days at room temperature.

Pathogen control strategies include separation of raw materials from RTE products, implementation of GMPs and controlled conditions, sanitary design of equipment and facilities, effective cleaning and sanitation procedures and controls, and an environmental pathogen

monitoring program are all parts of an effective pathogen control strategy in the food processing facility⁵⁸.

1.6.1 Foodborne Disease Outbreaks in the United States

Food can potential vehicle for food infectious or intoxications from pathogens of public health significance. A foodborne disease outbreak is the occurrence of two or more cases of a similar foodborne disease associated with the consumption of a common food¹³³. Foodborne outbreaks have been correlated with a wide range of foods commonly including fish, shellfish, dairy products, eggs, fruits and vegetables, meat and poultry, and grains¹³⁴. In the U.S., there are approximately 48 million cases of foodborne disease every year including 128,000 hospitalizations and 3,000 deaths¹³⁵. Thus, one out of six Americans become ill because of a foodborne illness each year. The estimated annual number of illnesses caused by the top five pathogens are shown in (Table 1- 2)¹³⁶.

Table 1- 2 Estimated Annual Number of Foodborne Illnesses in the United States Caused by Prevalent Pathogens

Pathogen	Estimated Number of Illnesses
Norovirus	5,461,731
<i>Salmonella nontyphoidal</i>	1,027,561
<i>Clostridium perfringens</i>	965,958
<i>Campylobacter</i>	845,024
<i>Staphylococcus aureus</i>	241,148

1.6.1.1 Foodborne Disease Outbreaks Associated with Pasteurized and Unpasteurized Dairy Products

Milk-borne outbreaks have decreased to less than 1% of all disease outbreaks compared to 25% in 1938 before pasteurization was widespread⁵⁸. This noticeable improvement could be primarily attributed to pasteurization and proper hygiene practices as well as to temperature control throughout milking, handling, shipping and storage of fresh milk and dairy products. Despite these encouraging statistics, the consumption of unpasteurized dairy continues to be a public health concern^{129,137,138}. Dairy products, such as cheese, are considered RTE foods, which do not require any additional heating or preparation to ensure safety before consumption. Cheese can become contaminated with pathogens from different sources and at several stages of processing. Several foodborne illness outbreaks associated with the consumption of contaminated pasteurized and unpasteurized milk and cheese products have been reported in the U.S., including illnesses caused by *Campylobacter jejuni*, *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes*^{137,139–141}.

L. monocytogenes has received special attention among other foodborne pathogens due to its high mortality rate. The FDA implemented a zero-tolerance policy for the presence of *L. monocytogenes* in RTE food or any other products¹⁴². Therefore, any RTE foods testing positive for *L. monocytogenes* is considered adulterated and cannot be legally distributed or sold. The first documented listeriosis outbreak in the U.S. was in California in 1985, which was associated with the consumption of Mexican-style cheeses, specifically queso fresco and cotija, produced from unpasteurized milk. This outbreak caused 142 illnesses, 28 deaths, and 20 fetal demises¹³⁹. *L. monocytogenes* still remains a foodborne pathogen of concern in dairy products. This pathogen also has been associated with numerous outbreaks within different food commodities, including

pre-cut celery, ice cream, frozen vegetables, cantaloupe, sprouts, caramel apples, and packaged salads^{143,144}.

In addition to numerous outbreaks, *L. monocytogenes* has caused multiple food recalls recently associated with different types of cheeses, as shown in (

Table 1- 3). The food recall is defined as a voluntary action taken by a manufacturer or a distributor to protect the public from food products that may cause health issues, such as illness or death. These products must be removed from the market after being adulterated or misbranded to avoid any public health problems, such as foodborne disease outbreaks¹⁴⁵.

Compared to other states where the sale of unpasteurized dairy products is legal; there were only two suspected foodborne outbreaks associated with dairy products in the state of Maine. The first was in 1998 associated with the consumption of unpasteurized milk, and the second was in 2001, which was associated with the consumption of pasteurized chocolate milk. The low incidence of foodborne illnesses may be due to the small scale of the Maine dairy industry as well as the strict state regulations regarding product safety and quality.

Table 1- 3 Recent Multi-state Recalls of Cheese Testing Positive for *Listeria monocytogenes* in the United States¹⁴⁶⁻¹⁵¹

Year	Implicated Cheese	Number of Illnesses
2017	Soft Raw Milk Cheese	8
2015	Soft Cheese	30
2014	Unaged Pasteurized Soft Cheese (Quesito casero)	5
2013	Semi-soft French Style Cheese	6
2012	Ricotta Salata Cheese	22

1.7 Regulations of Selling Unpasteurized Milk and Milk Products

In the late 19th century, public health reformers and activists highlighted milk in their agenda and the safety of the milk supply became a major concern was due to several illnesses and deaths of city urban residents including, children, after; drinking contaminated unpasteurized milk¹⁵². In 1920, milk regulations had reached all parts of the U.S., and in 1939, the U.S. Public Health Service had drafted the Model Milk Health Ordinance, which was adopted at the local level¹⁵². Since the 20th century, the regulation of unpasteurized milk has proved to be a great public health success in the United States. In 1973, the U.S. Food and Drug Administration (FDA) adopted a regulation which required that all milk for interstate sale must be pasteurized, and in 1982, the FDA initiated drafting regulations in banning the interstate commerce of unpasteurized milk and milk products¹⁵³. In 1987, the FDA activated the prohibition of the interstate sale or distribution of unpasteurized milk¹⁵³. Although the current FDA regulations (21 CFR 133) allow the interstate sale of cheeses made from unpasteurized milk after aging for at least 60 days at 35°F (1.67°C) or above, the safety of raw milk cheeses is still questioned¹⁰. There are thirty states in the U.S. which allow the intrastate sale of unpasteurized milk and milk products; however, only twelve of these states (including Maine), permit the sale of these products at retail stores⁶⁵.

1.8 Maine Dairy Industry

The dairy industry contributes more than \$570 million each year to Maine's economy, which generates more than \$25 million for state and municipal government taxes every year¹. The commercial retail sale of licensed, unpasteurized milk has been permitted since 1933 in Maine¹⁵⁴ and licensed producers of unpasteurized milk are permitted to sell their milk from the farm (direct to consumer), at retail stores, or at farmers' markets¹⁵⁵. A study by Welcomer et al. (2017)¹⁵⁶ mentioned that the number of licensed unpasteurized milk and cheese producers has increased

significantly from 15 and 21 producers in 2006 to 68 and 86 producers in 2016, respectively. The data of licensed dairy operations in their study were based on a personal communication with the Milk Quality Laboratory at the Maine Department of Agriculture, Conservation and Forestry. In Maine, the quantity of licensed operations is continuing to increase which places the state among the top artisanal cheese-making states in the U.S. and at the top of the Northeast¹⁵⁷.

1.9 Cheese Classification

Cheese is defined as a fermented milk product that possibly dates back to Neolithic times. Cheeses can be categorized based on the percentage of moisture on a fat-free basis (MFFB %) into soft, firm/semisoft, hard and extra hard cheeses as shown in (Table 1- 4).

Table 1- 4 Cheese Texture Classification Based on Firmness and Ripening Characteristics¹⁵⁸

According to Firmness		According to Principle Ripening Method
MFFB%	Classification	
< 51	Extra Hard	Ripened
49-56	Hard	Mold Ripened
54-69	Firm/Semi-Hard	Unripened/Fresh
> 67	Soft	In Brine

1.9.1 Hard Cheeses

Hard cheeses are characterized by having a moisture content which ranges between 30-45% and are exposed to higher pressures during pressing, which provides the finished product attributes based on the processing conditions¹⁵⁹. Hard cheese production has numerous steps, including renneting around 30°C, cutting the curd into smaller pieces and cooking the curd at 39-40°C, which allows for the release of whey. Some examples of hard cheeses include cheddar, Cantal, derby, ras, kefalotiri, and manchego. Cheddar is considered one of the most popular cheese

styles globally. It is produced on a large scale in the U.S., United Kingdom, Canada, New Zealand, Australia and Ireland. Cheddar is typically aged at 6-10°C for 3-4 months and up to 2 years or more, based on the ripening age desired¹⁵⁹.

1.9.2 Ripened Cheeses

Ripened cheeses are aged for a certain period of time, at a certain temperature and under certain humidity conditions. Throughout ripening, necessary biochemical and physical changes occur, which characterize the cheese's identity. For example, ripened firm/semi-hard cheeses include Saint-Paulin, Edam, gouda, provolone, tilsiter, danbo and havarti. While ripened hard examples include cheddar, emmental and samsø¹⁵⁸.

1.9.3 Soft Cheeses

Soft cheeses typically contain greater than 61% moisture, and 10-50% fat in the dry matter (FDM)¹⁶⁰. These types of cheeses are classified into four groups: un-ripened, mold-ripened, surface bacterial smear-ripened and pickled¹⁶⁰. These cheeses are ripened through the development of certain fungal cultures, such as *Penicillium camemberti* and *Penicillium roqueforti* in the interior and/or on the surface of the cheese^{158,159}. These two species of *Penicillium* contribute significantly to the appearance, texture and flavor development on the surface of mold-ripened and blue-veined cheeses¹⁶¹. Surface mold-ripened cheeses are soft varieties that have the characteristic white fungal mat on the cheese surface, such as brie, camembert and coulommiers. Blue-veined cheeses are identified by the blue-green growth of *P. roqueforti*, which gives the cheese the signature appearance and flavors¹⁶². These cheese flavors are generated by methyl ketones and other compounds, which result from free fatty acids being released by the action of the mold cultures¹⁶³.

1.10 Cheese Processing

In the earliest era of cheese manufacturing, cheesemakers counted on natural sources of lactic acid bacteria that would spontaneously ferment lactose to produce lactic acid. Currently, the cheesemaking process is usually standardized by the addition of starter cultures, including mesophilic (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*), and in certain styles of cheese, thermophilic cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Streptococcus thermophilus*)¹⁶⁴. The production of lactic acid by the action of these bacteria helps contribute to casein coagulation. Additionally, coagulation of casein is frequently supported by the addition of the enzyme chymosin, which is the active component of rennet¹⁶⁵. Rennet was initially extracted from the intestinal lining of milk-fed calves, which produces chymosin to assist in the digestion of milk. Chymosin cleaves the peptide bond between phenylalanine (Phe) 105 and methionine (Met) 106 in the κ -casein chain, which results in the clotting of milk during cheese processing¹⁶⁶. Additionally, it is responsible for the changes in texture, as well as the development in flavor throughout the ripening process¹⁶⁵. During the cheesemaking process, milk constituents are separated into two groups. Some are retained in the curd (casein and fat), while others are retained in the whey (such as water, lactose, peptides, other nitrogenous compounds, and minerals in the soluble form at a final cheese pH level of 4.6)¹⁶⁷. There are three common terms: artisan, farmstead-style, and specialty that are used to describe the way cheese can be produced¹⁶⁸. Farmstead-style cheese is cheese made by hand and the source of milk must be from the farmer's own herd¹⁶⁸.

Although there are numerous cheese varieties, cheese processing shares several common steps, which include ripening the milk, coagulation, cutting the curd, cooking, releasing the whey,

salting, molding, pressing, and aging the cheese for some varieties. Figure 1-2 illustrates the basic steps of farmstead-style cheese production¹⁶⁹.

1.10.1 Ripening

Ripening is the first step in cheese manufacturing after selecting the desired milk type (pasteurized or unpasteurized, whole, low fat, etc.). In the ripening process, milk is heated to the proper temperature before adding starter cultures. Heating the milk is achieved either by using stovetop or jacketed vat. Once the milk reaches the required temperature, the starter cultures can be added¹⁶⁹. Through the ripening stage, milk sugar (lactose) is transformed to lactic acid, as the added and innate milk cultures become activated in the warm milk. The increase of lactic acid in milk helps to facilitate the rennet action to clot milk, expel whey from the curd, to preserve the final cheese product and enhance flavor development¹⁵⁹. The accumulation of lactic acid lowers the curd pH level of curd which helps to produce an unfavorable environment for unwanted bacteria and helps to release whey (syneresis), as previously mentioned. Lactose is a polar disaccharide that is readily available in milk, and its concentration in cow's milk is consistently around 5%¹⁷⁰. Thus, the amount of both water and lactose will be equivalent in the whey after separating from the curds. Consequently, a small fraction, roughly 5% of water and lactose in the milk, is retained in the cheese¹⁷⁰.

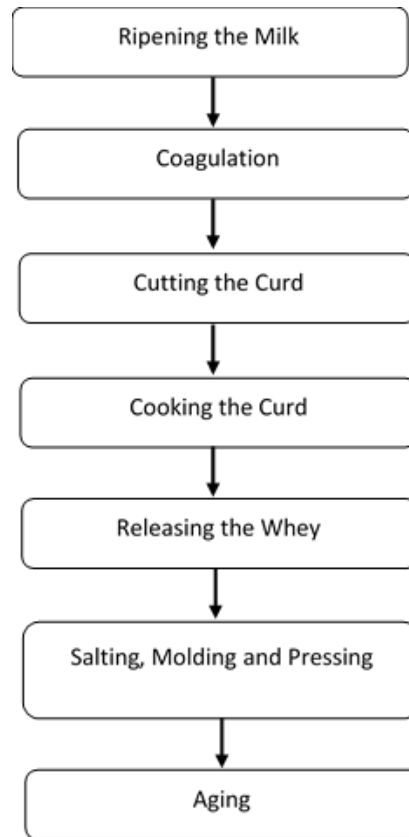


Figure 1- 2 The Cheesemaking Process

1.10.2 Coagulation

Coagulation is an essential step in curd formation, which involves the coagulation of the milk proteins (casein). There are three methods to coagulate milk proteins: acid, acid/heat and rennet coagulation.

1.10.2.1 Acid Coagulation

As previously mentioned, acidification can be achieved by the action of starter cultures (lactic acid bacteria LAB) or by directly adding acid to milk. When lactic acid or other acids are produced or added, the starting milk pH level is typically 6.7 and begins to descend towards the isoelectric point of milk protein (casein) which is 4.6. The aggregation of hydrogen ions reaches a

state of equilibrium on the polar surfaces of casein micelles and prevents them from interacting with water molecules¹⁷¹. This action forces the micelles to interact with each other and form micelle chains. These chains increase in length during the coagulation process and creates a three-dimensional net-like matrix, which entraps all water and solid components. This process transforms the milk from a liquid phase to a soft gel or coagulum. The coagulum is highly demineralized because most of the micellar calcium phosphate is dissolved by the accumulation of lactic acid. Generally, the demineralized casein does not have the capacity to expel much whey. For this reason, acid-coagulated cheeses are highly vulnerable to microbiological spoilage due to their higher water content (70-80% moisture), and the final pH value of 4.6 is particularly desirable for yeasts and molds. Thus, most of these high-moisture cheeses are consumed fresh since they have a much shorter shelf life compared to aged cheeses¹⁶⁷.

1.10.2.2 Acid/Heat Coagulation

In fresh milk, casein micelles are very heat-stable proteins and remain in a colloidal dispersion at temperatures up to 140°C³⁰. Moderately acidified milk (pH 5.4-6.2), either by lactic acid production or by the addition of an external acid, becomes sensitive to heat-induced coagulation at temperatures around 85°C. Since the whey proteins are heat susceptible, they will unfold and lose their capacity to interact with water molecules under acid/heat coagulation, which is called protein denaturation. Accompanying this process, the neutralization of the casein micelle polar surfaces by declining the pH level to the isoelectric point of 4.6 causes calcium-induced disorganization¹⁷². Consequently, denaturation of whey proteins prevents them from attaching to the micellar surfaces, and the casein micelles accumulate and entrap the fat globules. The resulting aggregates are called curds. The curd particles separate from the whey are able to be drained and pressed. Examples of acid/heat coagulated cheeses include ricotta, queso blanco and Turkish white

cheeses. These products typically have a high moisture content of approximately 50-80%^{167,172}. These cheeses are also more susceptible to microbial deterioration due to the high pH values and high moisture content.

1.10.2.3 Rennet Coagulation

Rennet is a proteolytic enzyme belonging to a group of aspartic proteases. There are two main sources of rennet: animal, which is from the stomach of milk fed calves¹⁶⁶ and microbial, produced by fungi such as *Rhizomucor* and *Cryphonectria*¹⁷³. Plant-derived enzymes, such as ficin from *Ficus* spp., papain from *Cracca papaya*, and Cardoon thistle (*Cynara cardunculus*) are also used in cheesemaking to clot milk proteins¹⁷⁴. Rennet coagulation includes two steps: the enzymatic and non-enzymatic phases¹⁷³. The enzymatic phase occurs by the hydrolysis of κ -casein, resulting in a caseinomacropeptide molecule. Rennet enzymes then effectively attach to the carbohydrate-rich, polar layer of caseinomacropeptides on the micellar surfaces. This process makes the interior of the micelles non-polar in milk, which is calcium-rich and activates the non-polar enzymatic phase. As in acid coagulation, the casein micelles lose the ability to interact with water molecules, and therefore, interact with each other forming micelle chains, which entraps milk solids. Rennet-coagulated cheeses have a higher calcium phosphate content and a higher buffering capacity, which helps to control the pH at the beginning stages of cheese ripening process. There are two major differences between acid and rennet coagulations. The curds from rennet coagulation are more tender and easier to expel whey from the formed curd than the from acid coagulation. The second difference is rennet coagulated curds can occur at a faster pace, within 30-60 minutes, at high pH levels (around 6.3-6.6); however, acid coagulation takes a longer time, about 5-48 hours to form a curd at low pH levels (approximately 4.6-4.8).

1.10.3 Cutting

Once the rennet is added and milk is transformed into solid curds, the curds are cut using a curd knife to help facilitate the separation of curd from the whey. The curd size is crucial, as smaller curd particles allows for a greater curd surface area. This will lead to the release of more whey, which will result in a lower moisture content and drier curd¹⁷⁵. In low-moisture cheese varieties, such as emmental and parmigiano reggiano, cutting the curd into rice-sized particles is required to help maximize whey expulsion. In high-moisture cheeses, the curd is usually cut into much larger pieces, reducing surface area and limiting whey expulsion to allow for more moisture retention in the curd¹⁷⁵.

1.10.4 Heating the Curds

The curds are slowly heated after cutting and then stirred. The heating process, accompanied by continuous stirring, enhances curd concentration and whey expulsion¹⁷⁵. Along with curd size, the temperature influences the moisture content of the final cheese, and the activity of LAB in producing lactic acid, which impacts the curd concentration and whey expulsion as the pH level drops. The heating temperature also affects curd demineralization and buffering capacity, as a result of its effect on the rate of lactic acid production by LAB¹⁶⁷. Normally, *L. lactis* subsp. *lactis* is inactivated at temperatures of 40°C (104°F) or above. Thus, any change in the temperature can have a significant influence on the rate of lactic acid production, especially at higher temperatures. This change can further impact the final product texture and moisture levels¹⁵⁹.

1.10.5 Releasing the Whey

After the curds have been heated and stirred, it is important to separate the curds from the whey. The curd particles then form a large matrix, which becomes the cheese. Syneresis is the

process of contraction of the curd particles due to the rearrangement of the para-casein network bonds. The rate of syneresis is influenced by the pressure gradient created in the gel network and by the flow resistance through the gel network. Several factors determine the rate and extent of the syneresis, such as temperature, pH, calcium chloride addition, curd firmness and cutting method¹⁷⁶⁻¹⁷⁹. Whey proteins dissipate in the milk liquid phase due to their folded structures, and the polar region of the amino acids opposing the water phase. For this reason, 5% of both lactose and whey proteins are expelled during syneresis in proportion to water throughout the manufacturing process of acid-rennet coagulated cheeses¹⁸⁰.

1.10.6 Molding and Pressing

Once the curds have been salted, they are molded and then pressed, depending on the cheese variety. Salt improves both the flavor and the cheese texture, as well as acting as a preservative by inhibiting the growth of unwanted bacteria. Salt also aids in moisture removal from the curds, causing them to shrink and drain more whey. Salting the cheese can be achieved by directly adding, rubbing or sprinkling salt onto the curd. The other salting method is brining, which is the immersion of cheese into a salt solution (brine). Cheese varieties that are commonly brined are feta and gouda cheeses¹⁶⁹.

Molding determines the cheese shape, and the amount of pressure and pressing time will influence the final texture of the cheese. Pressing helps to compress the curd and drain excess whey. Typically, cheese is pressed at a lower weight during a short period of time (10-15 minutes), and then the weight is increased gradually for at least 12 hours based on the desired final cheese texture¹⁶⁹.

1.10.7 Aging

As previously mentioned, the current FDA regulations (21 CFR section 133) permit the interstate sale of raw milk cheeses after aging for at least 60-days at 35°F (2°C) or above to ensure the microbial safety¹⁸¹. The aging or ripening process is the stage of developing the cheese flavor over time. This stage can take between a few days to six years depending on the cheese variety. Temperature and humidity are important factors during the aging process. Most cheese varieties have an optimal aging temperature between 46°F and 60°F with a relative humidity of 75%-95%. Flavor development is affected by the constant exchange of ripening gases that are released from the cheese, such as carbon dioxide and ammonia, as well as oxygen in the aging environment¹⁸². Usually, hard cheese varieties are aged at 55°F with a relative humidity of 65%-85%. The longer the hard cheeses are aged, the stronger the flavor development and the lower the moisture content¹⁶⁹.

Bacteria, molds and yeasts are present in cheese during the ripening process. Their contribution to ripening cheese is either direct, by their metabolism, or indirect by releasing enzymes into the cheese after autolysis. Cheese ripening is a complicated process that involves the action of both microbiological and biochemical changes. These changes affect the texture and flavor properties of the specific cheese variety¹⁸³. During the cheese ripening process, the microbiological changes include the lysis and death of starter culture cells (LAB), the growth of non-starter LAB (NSLAB), the growth of secondary microflora, such as *Propionibacterium freudenreichii* and the growth of molds. Some Gram-positive microflora also play an essential role in developing the flavor in some cheese varieties, such as smear cheeses¹⁸⁴.

During the aging process, the starter culture concentration can exceed 9-log colony forming units (CFU) per gram of cheese¹⁸⁵. Autolysis of the dying starter culture cells releases intracellular

enzymes, such as peptidases, and cellular components, such as sugars and nucleic acids, into the cheese¹⁸⁶. Meanwhile, NSLAB counts start to increase from 2-log CFU/g to reach approximately 7-8 log CFU/g after aging cheese for 3-9 months¹⁸⁷. The role of LAB, such as *L. lactis*, in developing cheese flavor is due to the proteolysis of casein by the release of proteinases¹⁸⁸. Another role of LAB during cheese ripening is amino acid catabolism. This is accomplished through the action of two enzyme classes: amino acid lyases and amino acid aminotransferases, which convert amino acids to flavor compounds¹⁸⁹. These biochemical changes during cheese ripening are divided into primary and secondary changes. The primary changes include glycolysis (as described in the ripening section), proteolysis and lipolysis, and the secondary changes include the metabolism of fatty and amino acids¹⁸⁴.

1.10.7.1 Proteolysis

Proteolysis is the degradation of proteins by the action of proteinase enzymes. Proteins are made up of amino acid chains, which are linked by peptide bonds. Proteins can lose their native conformation through the denaturation process either by enzymatic (rennet), chemical (acid) or physical action (by applying heat). Proteases can come from the milk, contamination with foodborne pathogens, added enzymes or bacteria, or the presence of somatic cells in the milk. The proteolysis of casein starts when rennet is added to the milk. The ideal action of rennet to clot the casein is under acidic conditions (pH level of 4.6). The second step of proteolysis is the formation of small peptides or amino acids by the action of the native enzymes (plasmin) in the milk or by the starter culture, which have direct impacts on cheese flavor¹⁹⁰. Proteolysis also contributes to cheese softening throughout the ripening processing by hydrolyzing the para-casein, reducing the water activity, and forming amino groups via hydrolysis of peptides^{190,191}.

1.10.7.2 Lipolysis

Lipolysis is the degradation of fats (lipids) by lipase enzymes. Triglycerides comprise 98% of the fat in milk¹⁹². The overall fat content in milk is 3.4%, and cheeses have roughly ten times more fat than milk. Milk contains approximately 65% saturated, 30% monounsaturated, and 5% polyunsaturated fatty acids¹⁹². For that reason, cheese is considered a high-caloric food containing high levels of saturated fatty acids (butyric, myristic, palmitic, and stearic acids). Likewise, cheeses are considered an abundant source of trans-fatty acids (conjugated linoleic acids) with typical values ranging between 8 and 18 mg/g of fat. Throughout the cheese ripening process, lipases degrade milk fat, essentially triglycerides, to short-chain fatty acids that might be unstable¹⁹³. Several factors, such as starter culture type, the duration of the ripening process, and cheesemaking conditions, might affect the lipolysis process. Short-chain fatty acids are important precursors for the production of volatile flavor compounds, which significantly contribute to flavor development and the aroma of many cheeses^{193,194}.

**CHAPTER 2 RETROSPECTIVE ANALYSIS OF MICROBIAL QUALITY OF
UNPASTEURIZED RETAIL MILK AND FLUID DAIRY PRODUCTS IN MAINE
BETWEEN 1998 AND 2016**

2.1 INTRODUCTION

Milk and dairy based-products are highly nutritious food commodities, which contribute more than \$570 million dollars to Maine's economy each year¹. Pasteurization is a proven thermal processing method to ensure the safety of these products. However, the increasing demand for unpasteurized dairy beverages has created a great deal of public health concern. In 1987 the Food and Drug Administration (FDA) prohibited the interstate distribution and sale of unpasteurized milk products, whereas intrastate sales were regulated at the state level¹⁵³. The sale of unpasteurized milk is allowed in thirty states, but only twelve of these states including Maine, permit the sale of these products at retail stores⁶⁵. In Maine, licensed unpasteurized milk producers are permitted to sell their products from the farm (direct-to-consumer), at retail stores, or at farmers' markets¹⁵⁵. Unpasteurized dairy distributors are required to obtain a Maine Milk commission license, which is regulated by the Maine Department of Agriculture, Conservation and Forestry, and is valid for one calendar year (Title 7, Section 2955 Maine Legislature Licenses)¹⁹⁵. Each dairy farm must be inspected annually to ensure the farm meets the state's standard requirements. According to the Pasteurized Milk Ordinance (PMO) and Maine Milk Rules, all dairy products produced in the state must be analyzed every six months⁵⁸.

Unpasteurized dairy products have been historically implicated in foodborne illness outbreaks^{153,196-199}. Most recently a 2016 multistate outbreak in Utah caused two illnesses as result of the consumption unpasteurized milk contaminated with *Campylobacter jejuni*²⁰⁰.

Cow and goat milk are the primary basis of most dairy products consumed by people today. Regardless of the source, each milk has a similar chemical composition including protein, fat, carbohydrate, vitamins, minerals, and water²⁰¹. However, the percentages of these constituents are influenced by several factors including stage of lactation, diet, genetics, and climatic conditions^{202,8}. Milk is an ideal medium for the growth of many microorganisms. Specifically, the relatively neutral pH (6.6-6.8) and extremely high water activity ($A_w = 0.99$) create a favorable environment for bacteria²⁰³. Microorganisms in milk originate from several sources such as the teat apex, milking equipment and tools, as well as the dairy farm environment^{31,66,204}. Subsequently, the initial total bacterial count of milk can differ significantly depending on factors such as hygienic practices, milk handling, and the animal's health status⁵. Therefore, it is critical that dairy farmers follow proper sanitation and milk storage practices in order to minimize sources of contamination^{58,5}.

Several microbiological tests have been established to evaluate the bacteriological acceptability of unpasteurized milk⁵⁸. More specifically, the standard plate and coliform counts (SPC and CC) are common procedures^{205,206}. Although not all bacteria enumerated using SPC is non-pathogenic, it can be an indicator of overall product quality. Coliform testing is an indication of sanitary quality and potential safety due to this class of bacteria being associated with fecal contamination. High coliform levels are usually caused by production problems, including poor hygiene practices and post-processing contamination^{207,208}. In the U.S., the legal maximum SPC limit for high quality "Grade A" raw milk is no more than 100,000 CFU/ml⁵⁸. The legal maximum coliform bacteria limit in "Grade A" pasteurized milk is 10 CFU/ml⁵⁸, which also applies to unpasteurized milk products.

A large pool of data regarding the microbial quality of unpasteurized dairy products in Maine has been generated from samples collected during Maine Department of Agriculture dairy inspections. Analysis of this data could provide valuable insight into certain factors that may affect the quality of these controversial products, such as potential seasonal effects, as well as provide information regarding industry trends. In this study, longitudinal microbiological testing data was obtained with permission from the Maine Department of Agriculture, Conservation and Forestry between 1998 and 2016, which include standard plate and coliform counts of unpasteurized retail fluid dairy products in Maine. By analyzing this data, we hoped to: (1) assess the overall quality and evaluate the cleanliness of unpasteurized retail dairy products in Maine, (2) investigate the effects of independent variables (season, year and animal species) on the quality of unpasteurized milk, (3) evaluate the microbial quality across types of unpasteurized retail dairy products (milk, light cream, heavy cream).

2.2 MATERIALS AND METHODS

2.2.1 Data

As previously mentioned, the dataset used in this study was obtained from the Maine Department of Agriculture, Conservation and Forestry, Milk Quality Laboratory in Augusta, Maine. The data set included SPC and CC data results for retail test samples of fluid unpasteurized goat and cow dairy products including milk, light cream, and heavy cream from licensed dairy farms between 1998 and 2016. The total number for cow and goat milk samples were 3087 and 717, respectively. Data were generated by serial dilution plating of retail samples onto 3M™ Petrifilm™ Aerobic and Coliform Count Plates. All samples were collected aseptically by state inspectors and then analyzed by trained laboratory technicians.

2.2.2 Descriptive Analyses

Statistical analyses were performed using IBM SPSS Statistics version 25. The SPC and CC data were log-transformed as follows: $\log_{10}(\text{original CFU/ml}+1)$ to compare the means²⁰⁹. The calendar year was divided into seasons with “fall” defined as September-November, “winter” as December-February, “spring” as March-May, and “summer” as June-August²¹⁰. In accordance with state regulations, $\text{SPC} \leq 50,000 \text{ CFU/ml}$ (4.7 log CFU/ml) and $\text{CC} \leq 10 \text{ CFU/ml}$ were used as the compliance threshold.

2.2.3 Statistical Analyses

An independent T-test was used to analyze statistical differences among the means. Due to large number of data set observations, non-parametric correlations (Spearman’s correlation coefficient) were used to detect any trends between milk quality standards (SPC and CC) and year. Chi-Square tests of independence were used to compare the continuous variables (SPC and CC) and categorical variables (seasons, product types, and animal species). Pearson’s Chi-Square was used to determine the associations between continuous and categorical variables. Standardized (Pearson) residuals were used to determine the statistical differences among seasons and among the dairy product types. *P* value of < 0.05 was set as the significance level.

2.3 RESULTS

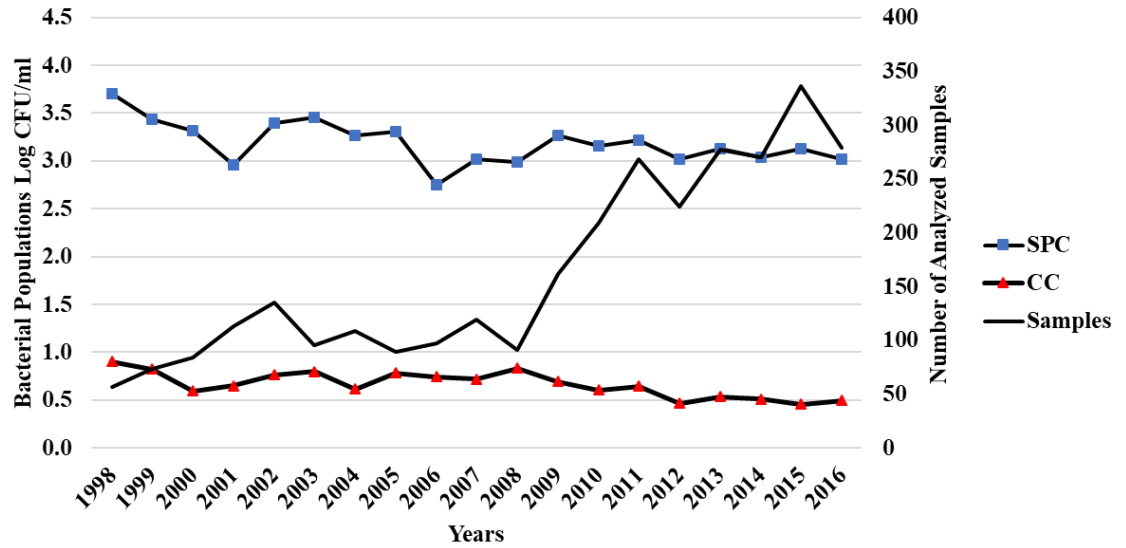
2.3.1 Bacterial Populations of Unpasteurized Retail Goat and Cow Milk Samples

Figure 2-1 displays the longitudinal bacterial population trends (SPC and CC), and the number of analyzed samples of both unpasteurized retail cow and goat milk samples. Overall, there were no statistically significant differences between the mean SPC totals for both cow and goat milk samples (3.14 and 3.12 log CFU/ml, respectively). However, the mean CC levels were

significantly ($p < 0.05$) higher (0.60 ± 0.01) in cow's milk samples, compared to goat milk (0.49 ± 0.03). In unpasteurized cow's milk samples, the highest mean SPC value was recorded in 1998 and the lowest in 2006 as shown in (Figure 2-1 A). Similarly, the highest mean CC values were observed in 1998 and 1999, whereas the lowest values were noted between 2012 and 2016. Clear trends were observed between bacterial populations and year for both dairy products. Figure 2-1A also demonstrates the increase in unpasteurized milk products analyzed during this time period. More specifically, the number of unpasteurized cow's milk samples increased overtime and were approximately four times higher compared to goat's milk samples analyzed in the same time period as shown in (Figure 2-1A).

Figure 2-1B shows bacterial populations (SPC and CC) in unpasteurized retail goat milk samples. In accordance with the results of unpasteurized retail cow's milk, the highest mean SPC and CC values were recorded in earlier years, while the lowest values were observed between 2010 and 2016. Overall, the mean SPC and CC populations in goat's milk samples decreased from 4.1 log CFU/ml in 1998 to 2.9 log CFU/ml over the duration of the data collection period, with variability in coliform counts also decreasing from 1.4 log CFU/ml to 0.4 log CFU/ml in recent years. Interesting to note, the number of retail samples for both dairy products were five times higher in 2016 compared to 1998. Spearman's correlation analysis demonstrated a significant weak negative correlation between milk quality results of both products and year.

A



B

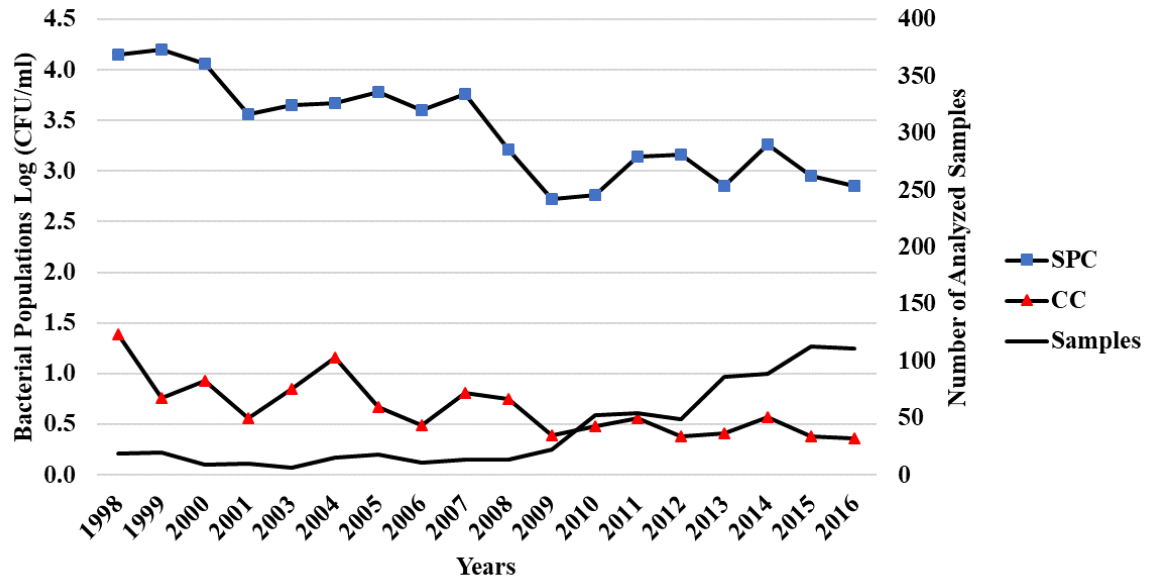


Figure 2-1 The Mean Bacterial Populations Values between 1998 and 2016 in Unpasteurized Retail (A): Cow's Milk and (B): Goat's Milk

Bacterial populations (Standard Plate Count SPC and Coliform Count CC) were expressed as means ± standard errors.

Total Samples of Cow's Milk n= 3087 and of Goat Milk n= 717.

The percentage of samples analyzed is displayed in Table 2-1. Overall, non-compliance was more likely to be the result of high CC rather than high SPC. No significant differences were associated among species and quality standards using Pearson Chi Square analysis.

Table 2-1 The Percentage of Compliant and Non-Compliant Bacterial Populations in Unpasteurized Retail Milk Samples.

Species	SPC ($p = 0.830$)*		CC ($p = 0.083$)*	
	% Compliant	% Non-Compliant	% Compliant	% Non-Compliant
Cow	90.30	9.70	74.10	25.90
Goat	90.10	9.90	77.30	22.70

*indicates Pearson Chi-Square correlation between species and SPC and CC is significant at level <0.05

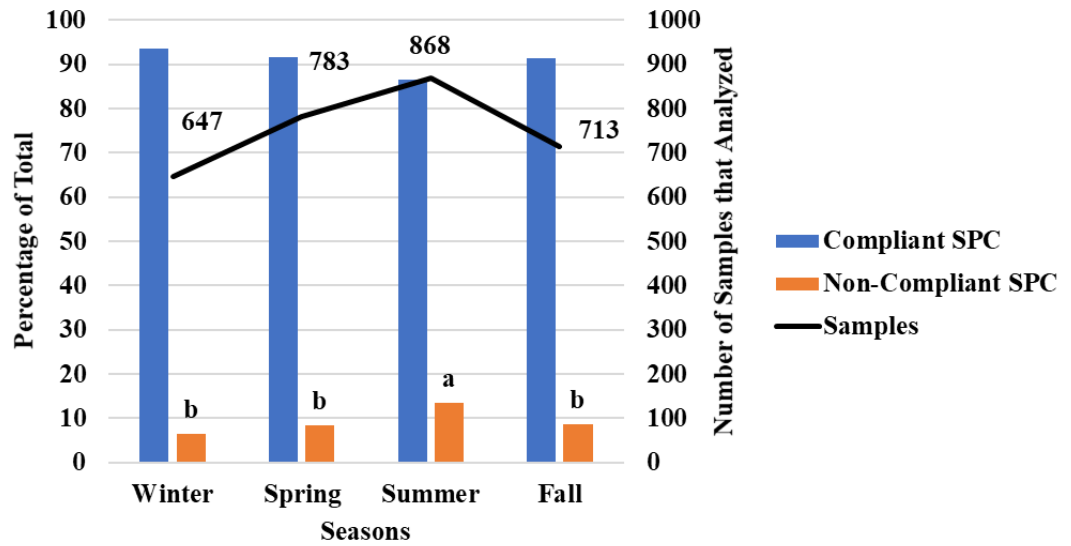
Cows' Milk $n = 3087$, Goats' Milk $n = 717$.

2.3.2 The Seasonal Effects on the Bacterial Populations of Unpasteurized Fluid Retail Cow and Goat Milk Samples

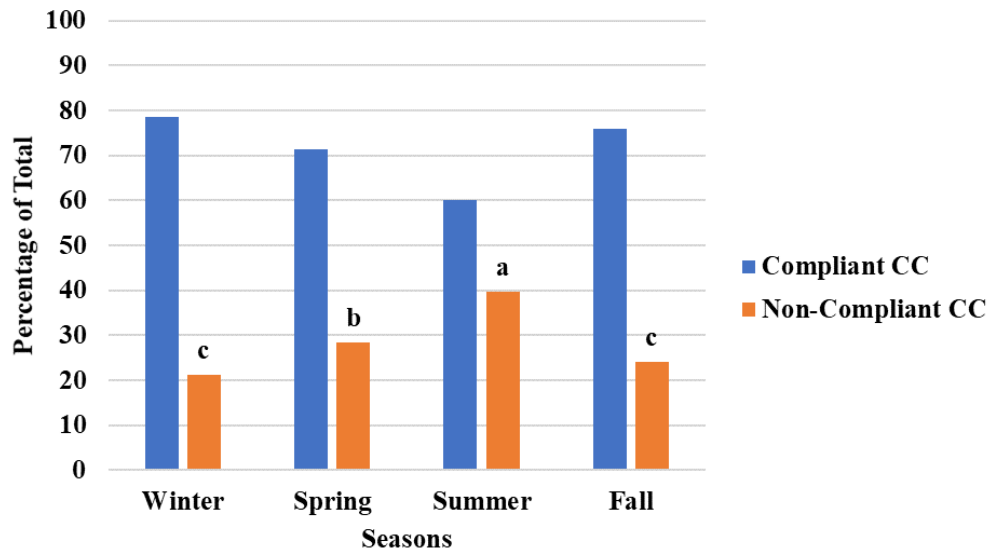
Figure 2-2 demonstrates the seasonal effects on the quality of retail samples of unpasteurized cow and goat's milk. As expected, higher percentages of non-compliant SPC and CC were observed in the summer for both dairy products. Across both cow and goat's milk, there were significant differences between non-compliant SPC and CC in summer compared to other seasons. Similarly, in cow's milk there were significant differences between non-compliant CC in summer and spring months, as well as between summer and both fall and winter months. Interestingly, all the submitted SPC goat's milk samples in the winter months were in compliance with the state regulations. Unlike cow's milk, the lower percentage of non-compliant CC samples were recorded in spring, which was significantly different than in fall and winter. The correlation between the prevalence of non-compliant SPC and CC in both products and seasonality was

determined, and the Pearson Chi-Square showed there were significant correlations between seasonality and both SPC and CC for both dairy products.

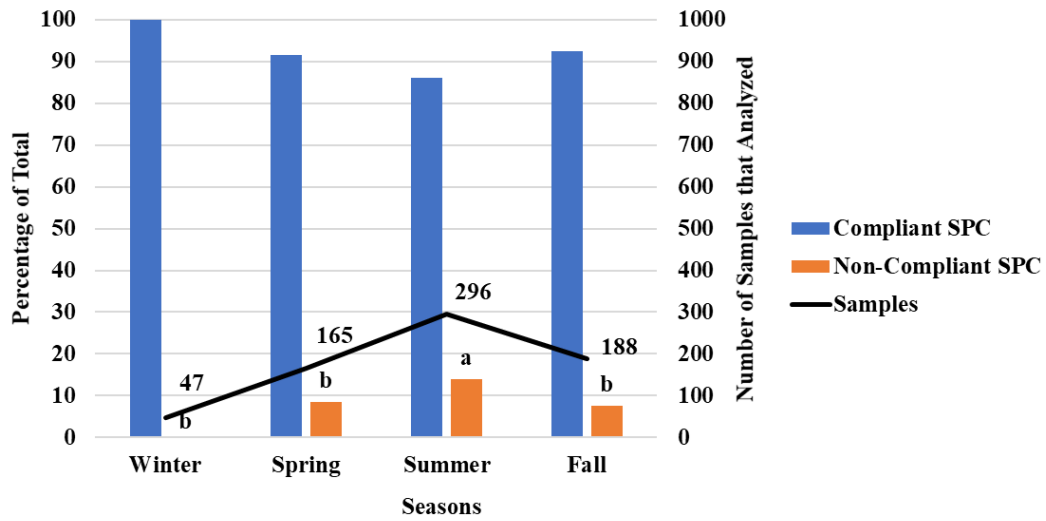
A



B



C



D

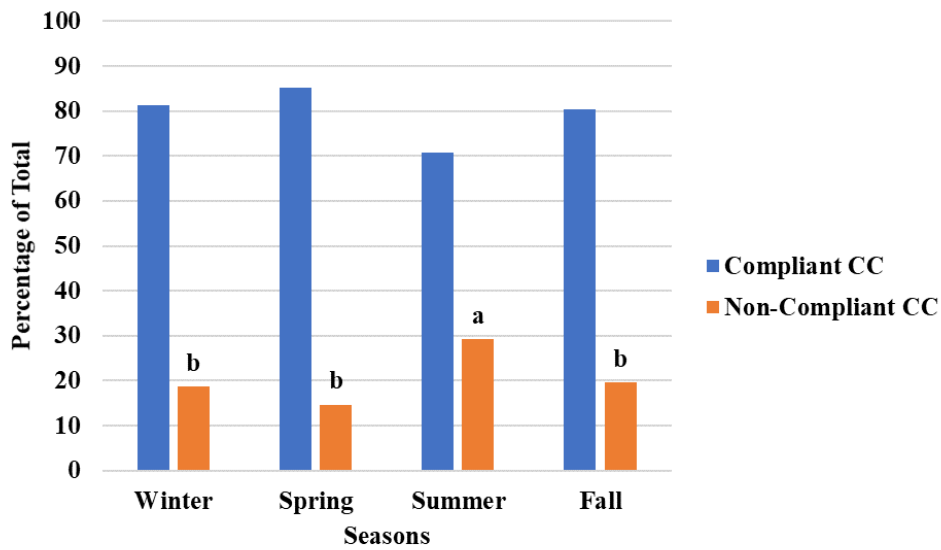


Figure 2-2 The Season Effects on Bacterial Populations in Unpasteurized Retail Cow's Milk (2A and 2B) and Goat's Milk (2C and 2D).

Compliant Standard Plate Counts (SPC): $\leq 50,000$ CFU/ml and Coliform Counts (CC): ≤ 10 CFU/ml. Non-compliant SPC $> 50,000$ CFU/ml and non-compliant CC > 10 CFU/ml.

Cow samples (Fall n=713, Winter n= 647, Spring n= 783, Summer n= 868) Goat samples (Fall n=188, Winter n= 47, Spring n= 165, Summer n= 296).

2.3.3 The Microbial Quality of other Unpasteurized Fluid Dairy Products

In addition to milk, other fluid products were analyzed including light and heavy cream as part of this data set as shown in Figure 2-3. Overall, a higher percentage of non-compliant samples were observed based on coliform count data compared to standard plate count results for all analyzed dairy products. Fluid milk had a significantly ($p < 0.05$) lower percentage of non-compliant SPC comparison to other dairy products. However, non-compliant CC percentages were consistent and not significantly different among all dairy samples.

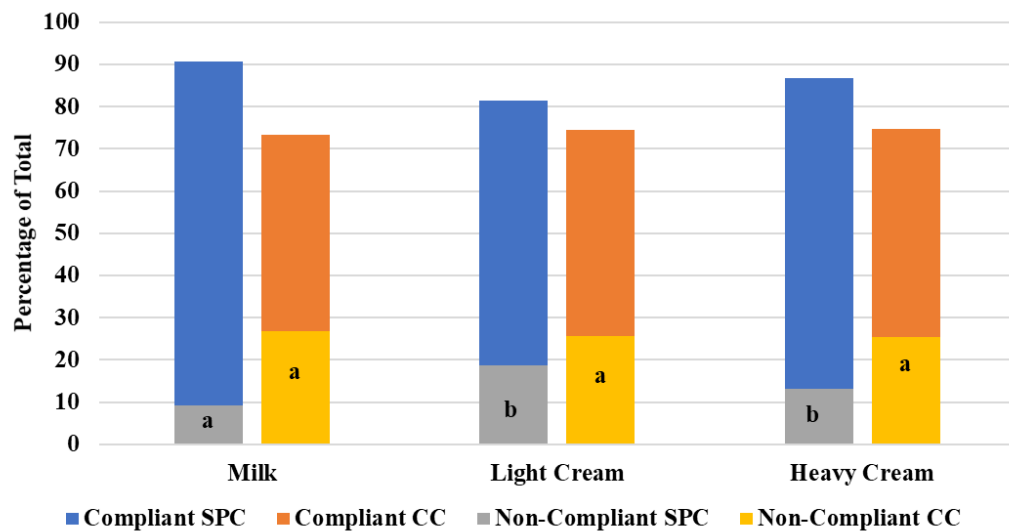


Figure 2-3 The Effects of Different Types of Unpasteurized Retail Cow's Milk Products on Bacterial Populations.

Chi-Square test was used, and significant differences were based on the value of standardized residuals.

Different letters across products were significantly different ($p < 0.05$).

Compliant Standard Plate Counts (SPC): $\leq 50,000$ CFU/ml and Coliform Counts (CC): ≤ 10 CFU/ml. Non-compliant SPC $> 50,000$ CFU/ml and non-compliant CC > 10 CFU/ml.

Milk samples $n = 3087$, Light cream $n = 43$, Heavy cream $n = 201$.

2.4 DISCUSSION

The microbiological community within milk is a significant indicator in evaluating the quality of dairy products. This study was designed to assess the microbial quality of unpasteurized retail dairy products based on retrospective testing data from retail samples produced by licensed dairy farms in Maine. The bacterial populations in Figures 1A and 1B revealed a decrease, thus the overall microbial quality has maintained over time even as there has been increase in retail samples over time. For example, the mean cow's milk SPC and CC declined significantly from 3.7 log CFU/ml and 0.90 log CFU/ml in 1998 to 3.02 log CFU/ml and 0.50 log CFU/ml in 2016, respectively. By analyzing this same data set, the number of dairy producers in 1998 was 18 and in 2016 was 53. This suggests that as market size increased the product quality has also improved likely as a result of the current inspection and licensing program. Furthermore, the increase in sample submissions may signify a greater demand for this product type in the market.

Due to the restrictions in many states on the sale of unpasteurized milk, there is a lack of long-term studies on the microbial quality of unpasteurized retail dairy products. However, a possible explanation for the reduction in SPC and CC observed in this study could be related to several factors, such as enhanced animal health, proper hygiene practices, and proper storage conditions. For example, Goldberg et al. (1991)²¹¹ found that microbial quality of Vermont bulk tank milk improved between 1985 (SPC 30,000 CFU/ml) and 1990 (SPC 23,000 CFU/ml). This was attributed to the implementation of an effective mastitis control program to maintain animals' health. In Maine, licensed dairies producing unpasteurized dairy products are required to submit at least four samples of their products during a consecutive six month period to ensure compliance with state regulations⁵⁸. However, if two of these four consecutive samples exceed the standard, the dairy farm concerned is required to send an additional sample for microbiological testing. Non-

compliance of the third sample will result in an immediate license suspension⁵⁸. Additionally, the annual inspection program is a second safe guard for preserving product quality. This is evident in the reduction of average microbial populations across the data set. Accordingly, the effectiveness of the state inspection program is apparent in the fact that there have be no reported outbreaks linked to unpasteurized dairy products in Maine within the past 20 years.

The SPC can be greatly influenced by specific groups of microorganisms, including thermotrophic, psychrotrophic, and environmental bacteria, such as *Streptococcus* spp. and coliforms^{212,213}. The correlation among different dairy quality standards, such as SPC and CC, and external factors including seasonality, sanitation, milk storage conditions, animal species, milk transport methods, and farm size has been well documented²¹³⁻²¹⁶. The results of this study showed that there were no significant correlation between SPC and CC in unpasteurized retail cow's milk; however, a significant ($p < 0.01$) but weak positive correlation between these standards was found in unpasteurized retail goat's milk. No significant ($p > 0.05$) correlation was observed between milk quality standards and animal species. Positive correlations between SPC and CC have been reported by multiple investigators, which suggests little differentiation of microbial populations in these samples^{217,218}. Researchers also have found that microbial counts and herd size were also positively correlated^{213,217}. The presence of coliforms in milk is generally related to animal cleanliness, dairy farm environment, and inadequate cleaning and sanitation of milking equipment²⁰⁶. Thus, the majority of compliance violations in our data set are a result of coliform bacterial presence. This suggests that there are incidences of potential contamination after milking and possibly farm sanitation issues.

High coliform counts (> 1000 CFU/ml) in unpasteurized milk may signify poor hygiene and sanitation practices, inadequate refrigeration, or the existence of coliform mastitis^{214,219,220}. In

the case of coliform mastitis, research has shown that *Escherichia coli* and *Klebsiella pneumoniae* have been isolated from cow's milk²²¹. An investigation by Van Kessel et al. (2004)²²² reported that 40% of bulk tank milk samples had coliform counts between 10-100 CFU/ml. These researchers also found that there were no differences in coliform counts from milk bulk tank samples across the U.S. regions (west, midwest, northeast, and southeast). High coliform counts could also be a result of undiagnosed mastitis in milking animals²²³. Although the results in this study showed there was no correlation between SPC and CC, these microbiological tests cannot be separated because SPC measures the overall milk quality, whereas CC typically measures the possible fecal, environmental, and post-process contamination of milk²²⁴. Conducting these tests simultaneously provides a more clear picture of the overall microbial profile. Moreover, it is important to note that sanitation practices may differ significantly in dairy operations intending to sell products without pasteurization (our data) compared to bulk tank milk that will be later pasteurized.

We further investigated the potential seasonal effects on milk quality. A possible explanation in higher non-compliant SPC and CC percentages in both cow and goat's milk samples in summer months compared to other seasons could be attributed to the variation of environmental temperatures in Maine. There was a significant correlation between milk sample quality and bacterial populations being higher when the outside temperatures are warmer in the summer. Previous studies attributed the increase in bacterial populations to the effect of temperatures changes between seasons, which is consistent with our findings. Harmon (1994)²²⁵ found that during the summer, the number of environmental bacteria in animal bedding material increased, which could be attributed to favorable temperatures and humidity for bacterial growth. Heat stress directly affects the susceptibility of the mammary host defenses against Gram-negative bacterial

infections, which causes an elevation in cases of clinical mastitis²²⁶. The occurrence of these infections and clinical mastitis are usually highest in summer months when the herds are confined²²⁶. Zucali et al. (2011)²²⁷ similarly observed higher numbers of SPC and CC during the warmer months (June and July) compared to the colder months (Dec, Jan, and Feb). Therefore, the lower temperatures in winter help to contribute to lower bacterial populations and thus, higher quality milk products during that time period.

In addition to product quality, seasonality has also proven to have an effect on milk production yields. Neciu et al. (2012)⁸ reported that an increase cows' milk production was observed in summer (June-September) compared to winter (December-February). The researchers attributed this variation to two potential factors. The first was food intake, which usually contains a higher percentage of green forage in the summer than in the winter months. The second factor was the relationship between exposure to day light. During the summer months, the animals are exposed to more day light, which stimulates the secretion of prolactin, which is the protein responsible for milk synthesis. As we observed in this study, the number of samples for both milk types were higher in summer compared to the winter months, which is consistent with the findings of Neciu et al. (2012)⁸.

The microbial quality of unpasteurized fluid retail dairy products was also investigated in this study. It should be noted that regardless of the product processing, all products had almost the same percentage of non-compliant CC, whereas unpasteurized fluid milk showed a lower percentage of non-compliant SPC compared to cream products. As previously mentioned, an increase in the total bacterial and coliform counts is a result of insufficient cleaning and sanitization, as well as post processing temperature abuse. In farm-produced cream, *Pseudomonas*, *Micrococcus* and yeasts are the most predominant microorganisms that develop sour, bitter, rancid,

and yeasty off flavors. Normally, the separation of cream from milk is achieved at 40-45°C, microbial growth is very likely to occur at this ideal temperature ranges if the cream is held at this temperature for a prolonged period of time²²⁸. Coliforms and lactococci also are responsible for slime formation in farm-produced cream. However, proper cooling and storage conditions (< 40°F) can limit the microbial growth in these products. Thus, improper cooling could be a potential source of increased coliforms that were noted in the cream data set. As reported by the literature, the microflora of cream-based products should similar as the microbial counts of full-fat whole milk²²⁹. Therefore, the processing of cream products has an important role in the quality of dairy products. The sanitation of the separation equipment could also affect the bacterial counts of these milk products²³⁰.

2.5 CONCLUSIONS

The interest in locally produced and minimally processed foods, such as unpasteurized dairy products has increased in the U.S.²³¹. This is especially true in Maine where the sale and distribution of unpasteurized dairy products have been permitted at the retail level since 1933. There will always be a certain degree of concern for consuming these products as a result of the inherent properties of a minimally processed product. However, the results of this study suggest that the overall quality of an expanding market of unpasteurized dairy products in the state of Maine remained constant, most likely as a result of the current state inspection program's adequacy in safe guarding these commodities. Furthermore, fecal contamination (as illustrated by the coliform count data) as well as seasonality, are major contributing factors to food safety risks associated with dairy. However, maintaining and monitoring a consistent and effective sanitation program will help to maintain low microbial counts throughout the year. We can conclude from this study that the overall quality of unpasteurized dairy products has remained relatively constant

despite the local market expansion of these products. Furthermore, higher non-compliant samples were detected during the summer months than in winter, and coliforms were the overall cause of non-compliant status in all analyzed products. Thus, more attention should be paid towards sanitation to minimize sources of possible fecal contamination, especially during the summer.

Overall, the data shows that Maine has high quality unpasteurized milk products with a compliance of 90% for SPC for both cow and goat's milk, and also greater than 70% compliance of coliform counts, and no reported foodborne illness outbreaks within the past 20 years.

**CHAPTER 3 THE DYNAMIC BEHAVIOR AND SURVIVAL OF *LISTERIA*
MONOCYTOGENES AND SHIGATOXIGENIC *ESCHERICHIA COLI* DURING THE
AGING OF FARMSTEAD-STYLE CHEESE**

3.1 INTRODUCTION

Foodborne disease outbreaks have been associated with a wide range of foods, including unpasteurized (raw) and pasteurized milk and dairy products^{129,137,232,233}. Raw milk can become contaminated with pathogens through several sources, including fecal contamination, udder infection (mastitis), direct passage from the cow's blood to the milk, and contamination from human skin during milking^{5,234}. In an effort to minimize outbreaks, the US Food and Drug Administration (FDA) prohibited the interstate sale or distribution of raw milk in 1987¹⁵³. However, current FDA regulations allows the interstate sale of cheeses made from unpasteurized milk after aging cheese for at least 60 days at 35°F (1.67°C) or above²³⁵. Cheese is considered a ready-to-eat (RTE) food product, which does not require any additional heat processing to ensure its safety before consumption. Therefore, potential foodborne illness risks can increase as a result from poor sanitation practices and cross contamination from the dairy environment. Although several bacteria have been implicated in dairy-related foodborne illness outbreaks, *Listeria monocytogenes* and *Escherichia coli* are two prominent bacteria that have been associated with contaminated dairy products from past outbreaks. In the United States, the first recorded *E. coli* (enterotoxigenic *E. coli* O27:H20) outbreak, associated with the consumption of imported semisoft cheese, was in Washington D.C. in 1983²³⁶, while the first recorded outbreak of *L. monocytogenes*, was correlated with Mexican-style cheeses queso fresco and cotija in California in 1985¹³⁹. These outbreaks established pathogenic *E. coli* and *L. monocytogenes* as significant threats to the dairy

food industry. The FDA has established a zero-tolerance policy at any detectable level for *L. monocytogenes* due to a high mortality rate¹⁴², and *E. coli* has a low potential infectious dose (10-100 CFU)²³⁷ to susceptible populations.

Shiga toxinogenic *E. coli* (STEC) is considered a subgroup of Shiga toxin-producing *E. coli* (STEC). *E. coli* strains are Gram-negative, rod-shaped, facultative anaerobes, and predominant in the gastrointestinal tract of healthy mammals²³⁸. Illness from this subgroup is characterized by diarrhea or hemorrhagic colitis, which progresses to hemolytic uremic syndrome (HUS), and can lead to acute renal failure in children^{112,239}. Although *E. coli* O157:H7 is considered the major strain of STEC and accounts for most global infections, other non-O157 STEC serotypes, including O26, O111, O121, O103, O45, and O145, have also been isolated from clinical infections^{119,120,239}. The two *E. coli* serotypes that have been most frequently implicated in dairy product outbreaks are *E. coli* O157:H7 and *E. coli* O26:H1¹⁴. Cattle are among the primary reservoir of STEC strain carriers; however, bovine typically do not express any clinical symptoms²⁴⁰. Furthermore, the ability of STEC strains to survive at low temperatures, and can promote an acid tolerance response in mildly acidic pH environments, makes these *E. coli* serotypes an important health concern²⁴¹.

Listeriosis is a severe disease with a relatively high mortality rate compared to other foodborne illnesses caused by other pathogens, such as *E. coli* O157:H7 and *Salmonella*. This disease is caused by *L. monocytogenes* a Gram-positive, non-sporeforming, facultative anaerobe that is rod-shaped and can survive and reproduce in a wide range of environmental conditions²⁴². The primary risk to cheese safety is usually associated with environmental contamination after pasteurization from either the cheese processing facility or during cheese aging^{243,244}. Due to the psychrotrophic nature, and the acid tolerance of *L. monocytogenes* to survive and continue to grow

at refrigeration temperatures²⁴⁵, this bacterium is considered the largest foodborne pathogen threat to refrigerated RTE foods.

Several studies have investigated the behavior of *L. monocytogenes* and *E. coli* during the manufacturing and aging of different types of cheeses made from pasteurized and unpasteurized milk^{12,20,241,246-248}. The survival of foodborne pathogens in cheese is dependent on several factors, including cheese making procedures, cheese type, starter cultures, pH, aging temperatures, and the bacterial outer membrane composition²⁴⁹⁻²⁵¹. Most importantly, the starter cultures (lactic acid bacteria LAB) produce antimicrobial compounds, which are imperative to ensure food safety by suppressing the growth of foodborne pathogens^{252,253}. In laboratory studies, experimental inoculation methods, and the levels of initial inoculum both play a significant role in the pathogen's population dynamics. Previous studies have focused on the survival of *E. coli* O157:H7 during the manufacturing of different types of unpasteurized cheese, whereas other non-O157 *E. coli* serotypes survival has not been validated. Therefore, additional studies are required to understand the capabilities of these pathogen strains within this food matrix.

Cheesemakers may decide to use a variety of temperatures during aging for a number of reasons, including flavor and aroma development, desirable mold growth, texture enhancement, and ultimately product safety. In this study, the survival of two relevant pathogens (*L. monocytogenes* and STEC) was monitored during aging at different temperatures (4°C, 10°C and 22°C) chosen for feasibility purposes for small cheesemaking operations. The objectives presented in this study were: (1) to investigate the behavior of *L. monocytogenes* and STEC during the manufacture of unpasteurized farmstead-style cheese, (2) to study the survival of these pathogens during aging, and (3) to examine the effects of three applicable aging temperatures on the survival of these pathogens.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

Shigatoxigenic *E. coli* (STEC) O26:H11 EH1535 (ATCC BAA-1635) and O111:H8 (ATCC BAA-184), and *L. monocytogenes* serotype 4b (ATCC 19115) and serotype 1 (ATCC 19111) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These bacterial strains were maintained in the Pathogenic Microbiology Laboratory, School of Food and Agriculture, at the University of Maine (Orono, Maine, USA). Frozen stock strains were held at -80°C in glycerol prior to use. Strains were added to 10 ml of trypticase soy broth (TSB) (Acumedia, Lansing, MI, USA) supplemented with 0.6% yeast extract (IBM Scientific, Peosta, IA, USA) to begin the inoculum preparation.

3.2.2 Inoculum Preparation

Bacterial strains were sub-cultured individually twice in 10 ml of TSB supplemented with 0.6% yeast extract. The inoculated TSB tubes were incubated for 24±2 hours at 37±1°C for STEC and for 24-48 h at 30±1°C for *L. monocytogenes*. Overnight bacterial cultures were centrifuged twice for 10 minutes at 10,000 X *g* at 20°C using an Eppendorf Centrifuge 5810R (Hamburg, Germany) and washed with 10 ml of 0.1% peptone water (BD Bacto™, Sparks, MD, USA), and then re-suspended with 10 ml of 0.1% peptone water. A cocktail of STEC and *L. monocytogenes* strains was added to 3 gallons of unpasteurized milk to provide an initial inoculum of 4.5 CFU/ml of each species.

3.2.3 Starter Culture

A freeze-dried, commercial starter culture (Mesophilic C101) composed of lactose, *Lactococcus lactis* subsp. *lactis*, and *Lactococcus lactis* subsp. *cremoris* was purchased from New

England Cheesemaking Supply Company (South Deerfield, MA, USA). The starter culture was stored at -20°C until further use.

3.2.4 Milk Collection

Unpasteurized milk was collected from J. Franklin Witter Teaching and Research Center at the University of Maine (Old Town, Maine, USA) on the day of milking and transported in clean and sanitized food grade containers. Raw milk was refrigerated for 2-4 hours at 4°C until further use. Mesophilic standard plate counts (SPCs) were determined by serially diluting milk in 0.1% peptone water. A series of dilutions were plated onto trypticase soy agar (TSA; Alpha Biosciences, Baltimore, MD, USA) plates in duplicate and incubated for 24±2 hours at 37±1°C before enumeration.

3.2.5 Cheese Manufacturing

A farmstead-style cheese recipe was selected and used for the cheese preparation¹⁶⁹. Cheese was made in three-gallon batch sizes in stainless steel stockpots. An uninoculated batch was produced as the control, and another batch was inoculated, as previously described. The milk was heated to 32°C using a single electric burner (Mainstays; China), and the temperature was monitored during the heating process using a glass thermometer. The thermometer was inserted at approximately half of the milk depth and clipped to the edge of the stockpot. A sterile stainless steel skimmer was used to stir the milk intermittently. A commercial starter culture (1.5 g/3 gallons) was added once the temperature reached 32°C. The milk was held at 32°C for 60 minutes using a bacteriological incubator. Single strength liquid rennet (3.5 g/3 gallons; R7 liquid animal rennet, South Deerfield, MA, USA) was added and milk was lightly stirred using the sterile stainless steel skimmer. The milk was incubated for an additional 45 minutes at 32°C using the incubator. After coagulation, the curds were cut into small pieces (1-inch cubes) using a sterile,

stainless steel knife. After cutting the curd, the curds were cooked until the temperature reached 39°C within approximately 7-10 minutes accompanied with intermittent stirring. The curds were then drained from the whey using a sterile stainless steel skimmer, and 30 g of pickling salt (Canning and Pickling Salt, Morton Salt, Inc, Chicago, IL, USA) were added and mixed thoroughly into the curd using the stainless-steel skimmer. Cheese curds were transferred into custom-made, stainless steel molds (81.9 cm³) and pressed at room temperature in a stainless steel Dutch style cheese press. The curds of both inoculated and control cheeses were pressed side by side in two separate presses for 30 min using 35 pounds of total pressure for each cheese, and then 45 pounds pressure was applied to the cheese samples for 18 hours. The final cheese weight was approximately 3 pounds. The cheesemaking process was repeated three times.

3.2.6 Vacuum Packaging

After pressing, a sterilized knife was used to cut the cheese samples into small pieces (approximately one cubic inch, 18 g/piece) for individual microbiological analysis. Each cheese sample was packed into individual sterile, polyethylene bags (Weber Scientific, Hamilton, NJ, USA) and vacuum packaged using a VACMASTER VP 210 (Kansas City, MO, USA).

3.2.7 Cheese Samples and Aging Conditions

Vacuum packaged cheese samples were aged simultaneously at three temperatures: 4°C using a household refrigerator (Hotpoint, Louisville, Ky, USA), 10°C using a Thermoelectric Wine Cooler (Intertek SC-08B), and 22°C using a bacteriological incubator (Benchmark Scientific H2265-HC; Sayreville, NJ, USA). Cheese samples (control and inoculated cheeses for each temperature) were analyzed weekly for 84 days.

3.2.8 Bacterial Enumeration and Detection

For microbiological analyses, each vacuum packaged cheese sample was diluted in warm (45°C), sterile 2% (w/vol) sodium citrate dihydrate solution (Fisher Chemical, Fair Lawn, NJ, US), and homogenized for 2-3 minutes using a sterile Waring Commercial Blender 700S (Torrington, Connecticut, USA). For direct enumeration, the cheese homogenate was serially diluted in 0.1% peptone water, and spread-plated in duplicate onto deMan, Rogosa and Sharpe (MRS) medium (Alpha Biosciences, Baltimore, MD, USA), sorbitol MacConkey Agar (sMAC; Acumedia, Lansing, MI, USA) and Modified Oxford Agar (MOX; Alpha Biosciences, Baltimore, MD, USA) plates supplemented with modified oxford antibiotic supplement (BD Difco; Sparks, MD, USA) (FDA BAM, 2017). Plates were incubated at 35±1°C, 37±1°C and 30±1°C, respectively, for 24-48 hours before enumeration. When the population of each studied pathogen was near or below the detection limit (100 CFU/g), an enrichment procedure was used. The cheese samples were diluted in 2X 2% sterile sodium citrate dihydrate, homogenized for 2-3 minutes, and then a solution of 2X selective enrichment medium was added. *E. coli* (EC) medium (Alpha Biosciences, Baltimore, MD, USA) was used to enrich samples for *E. coli* detection, and Buffered Listeria Enrichment Broth (Alpha Bioscience, Baltimore, MD, USA) was used to enrich samples for *L. monocytogenes* detection²⁵⁴. Enrichments were incubated as previously described. After incubation, aliquots of enriched samples were plated onto selective medium and the plates were incubated under the same conditions for both pathogens before the plates were examined for typical morphology colonies.

3.2.9 Enumeration of Fungi

Fungi were enumerated at the end of aging (day 84) using acidified potato dextrose agar APDA (Alpha Bioscience, Baltimore, MD, USA). Tartaric acid (Sigma-Aldrich; St. Louis, MO, USA) was prepared in distilled water to a concentration of 10%, filtered and then added to PDA after autoclaving. The APDA plates were incubated at room temperature 22-25°C for 5 days prior to enumeration.

3.2.10 Measuring the pH and Water Activity Levels

The water activity and pH levels of the uninoculated controls were measured at each sampling point. To determine the water activity, a cross-sectional piece of cheese approximately 1.5-2.0 g was cut using a sterile knife. The AquaLab Pre Water Activity Meter, (Pullman, WA, USA) was calibrated prior to use using AquaLab standards. A HANNA pH meter (Woonsocket, RI, USA) was used to measure the pH. A cross section of the cheese sample was cut and the FC 2020 pH Edge electrode with conical glass tip (Woonsocket, RI, USA) was placed into the sample to determine the pH level. The water activity and pH measurements were conducted in duplicate.

3.2.11 Texture Profile Analysis (TPA)

The control cheese sample texture was analyzed on days 0 and 84 for each aging temperature using a compression test with a TAXT2i texture analyzer (Texture Technologies Inc.; Scarsdale, NY, USA). The texture analyzer was calibrated using a 5,000 g standard weight. Cheese samples were placed on a petri dish and placed onto the texture analyzer platform. TPA was performed using a 12.7 mm (½ inch) cylindrical probe, at a 2 mm/s test speed. Force (g), area (g × s), distance (mm) between peak heights, and time (s) were recorded using the texture analyzer

software (Exponent 32, version 5.0, 6.0, 2010, TAXT2i Texture Technologies Inc., Scarsdale, NY, USA) to calculate TPA. Values were averaged for (n=3) cheese samples per treatment replicate.

3.2.12 Statistical Analysis

Data analysis was performed using IBM SPSS Statistics version 25 (Orem, UT). Bacterial populations of *E. coli*, *L. monocytogenes* and LAB were expressed as CFU/ml or g and log-transformed before analysis as follows: $\log_{10}(\text{original CFU/ml}+1)^{209}$. Physicochemical analyses (pH, water activity, and texture) were averaged for each time point. To monitor the behavior of pathogens during the cheesemaking process, and to investigate the effects of temperatures during aging on pathogen counts and physiochemical parameters, one-way analysis of variance (ANOVA) was used to determine statistically significant differences among treatments followed by Tukey's honest significant difference (HSD) for mean separation. *P* values < 0.05 were considered as statistically significant. Data were expressed as mean \pm standard deviation of the three independent trials.

3.3 RESULTS

3.3.1 Milk

The unpasteurized cow's milk was plated prior to making cheese and the average SPC was 1×10^3 CFU/ml (3 log CFU/ml) after 24 hours of incubation on TSA plates at 37°C. This average was within the state legal limit $\leq 50,000$ CFU/ml (4.7 log CFU/ml).

3.3.2 The Behavior of Pathogens during Farmstead-style Cheese Manufacturing

Figure 3-1 shows the behavior of *E. coli* and *L. monocytogenes* during the cheesemaking process. Throughout the cheesemaking process, there was no significant ($p > 0.05$) changes in the

mean *E. coli* population among all production steps, although *E. coli* appeared to be at the lowest levels during the cheese coagulum phase and in the whey. However, there was a significantly lower population of *L. monocytogenes* detected in the whey (3.05 ± 0.01 log CFU/ml) when compared to milk and cut curd as shown in Figure .

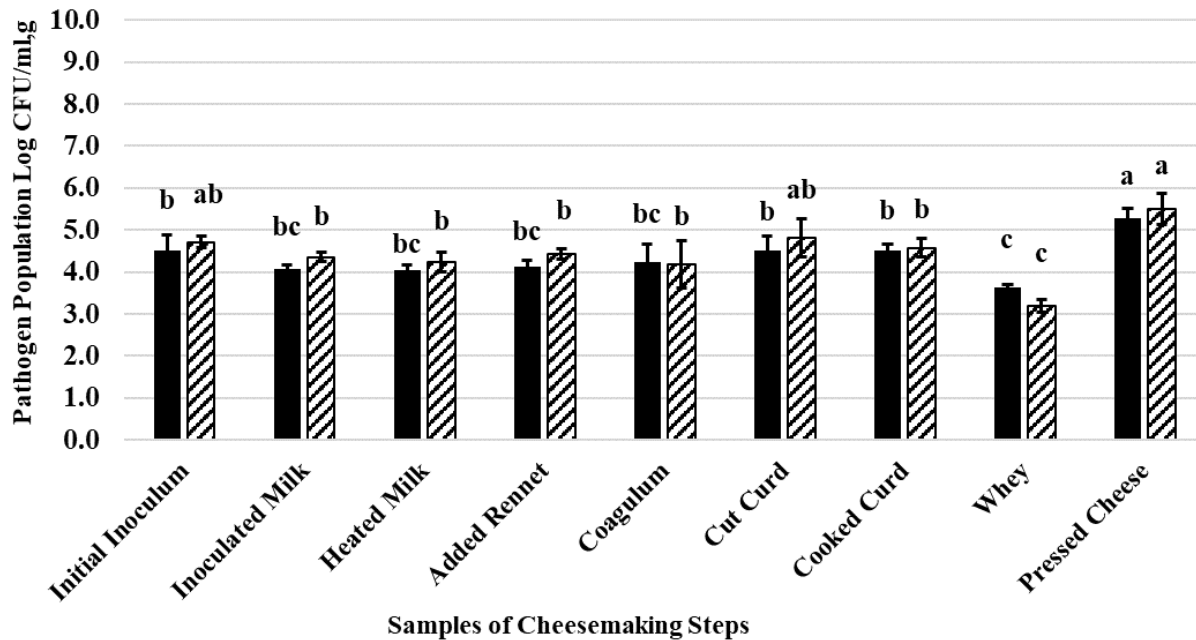


Figure 3-1 Behavior of *E. coli* and *L. monocytogenes* during Cheese Manufacturing

Pathogen population data were expressed as mean \pm standard errors.

Different letters were significantly different ($p < 0.05$), ANOVA (Tukey's HSD) within bacterial species.

Initial inoculum of each pathogen was 4.5 log CFU/ml.

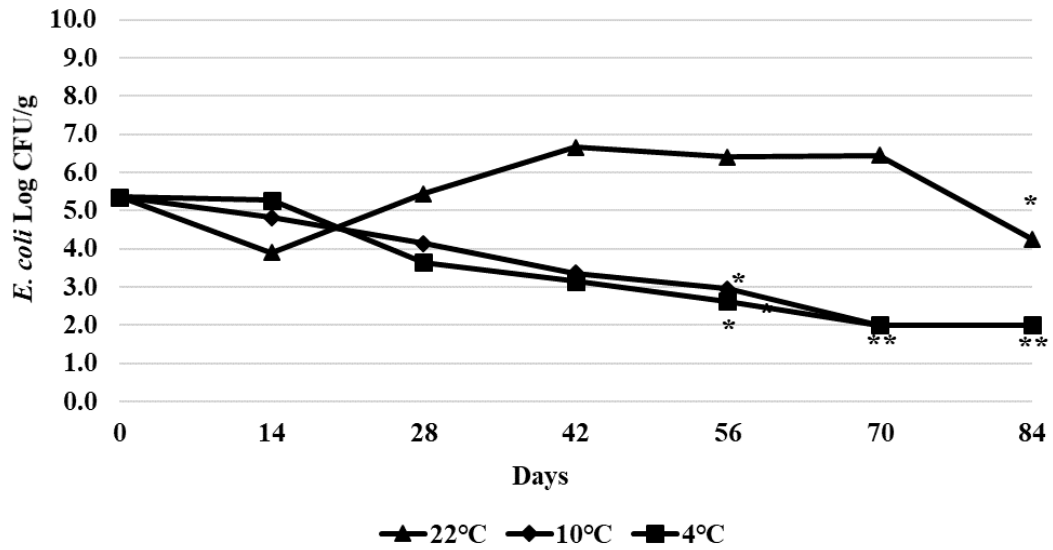
n=3.

3.3.3 The Survival of Pathogens during the Aging of Unpasteurized Cheese

Figure 3-2 represents the effects of temperature (4°C, 10°C and 22°C) on the survival of *E. coli* and *L. monocytogenes* during the aging of artificially inoculated unpasteurized cheese.

Aging the cheese at both 4°C and 10°C resulted in a significant reduction of *E. coli* counts; however, aging at 22°C resulted in a significant increase in counts, as shown in Figure 3-2 A. The *L. monocytogenes* counts were also significantly reduced at 4°C; however, at both 10°C and 22°C, *L. monocytogenes* populations significantly increased as shown in Figure 3-2 B. The enrichment results showed that when cheese was aged at 4°C, *E. coli* was reduced to <100 CFU/g after 63 days, while in cheese aged at 10°C, *E. coli* was reduced to <100 CFU/g at day 70 (Figure 3-2 A). Similarly, the enrichment results revealed that when cheese was aged at 4°C, *L. monocytogenes* was reduced to <100 CFU/g at day 70 (Figure 3-2 B). Overall, aging the cheese at lower temperatures (4°C and 10°C) was effective in reducing the bacterial populations of *E. coli*, while *L. monocytogenes* populations were successfully reduced at 4°C.

A



B

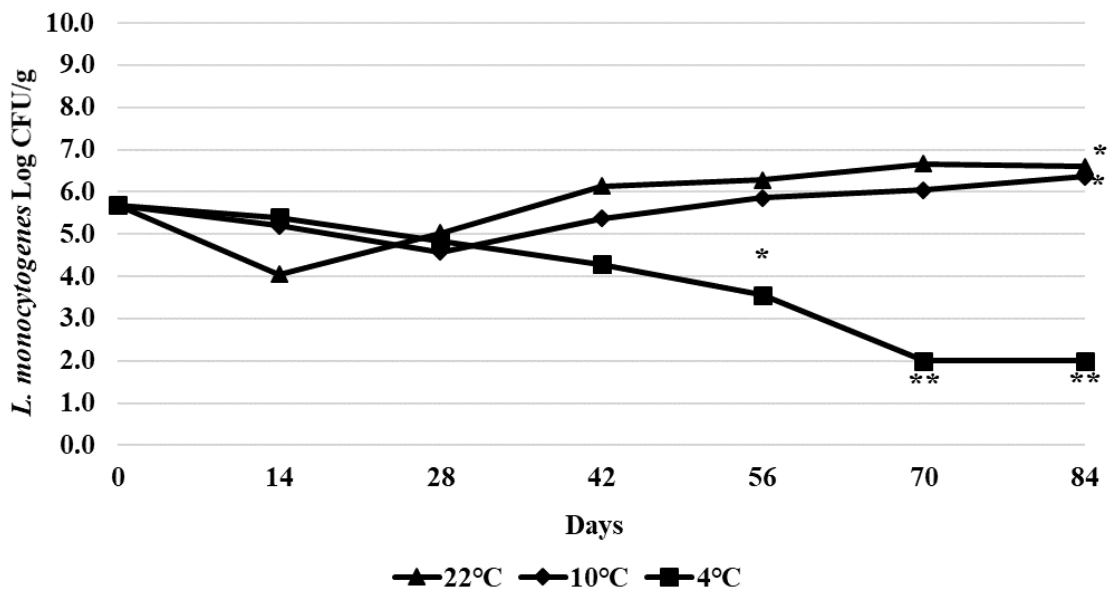


Figure 3-2 The Effect of Temperature on the Survival of *E. coli* (A) and *L. monocytogenes* (B) during Cheese Manufacturing

Data expressed as mean Log CFU/g \pm standard deviation.

*: represents significant differences within treatments when compared to day zero.

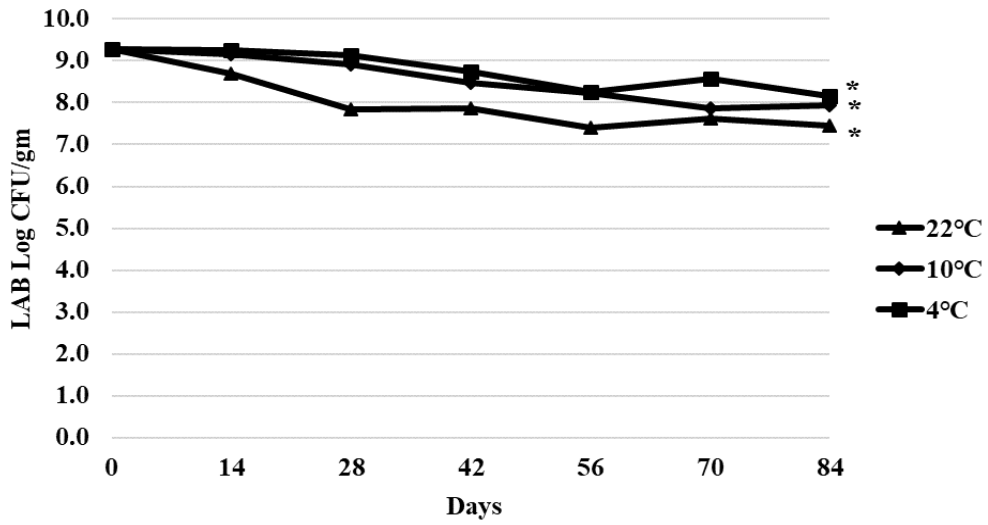
** : indicates below the detection limit (100 CFU/g).

n=3.

3.3.4 The Effects of Temperature on Lactic Acid Bacteria (LAB) Populations During the Aging Process of Unpasteurized Cheese

The LAB counts were plated for both control and inoculated cheese samples aged at different temperatures (4°C, 10°C and 22°C). Figure 3-3 shows the effects of the mentioned temperatures on the LAB counts over time. As shown in (Figure 3-3 A and B), the rate in the reduction of beneficial LAB was faster at 22°C compared to 4 and 10°C during the aging process. Overall, aging cheese at the lowest temperature (4°C) was more effective in maintaining the beneficial LAB counts in both control and inoculated cheese samples over aging time.

A



B

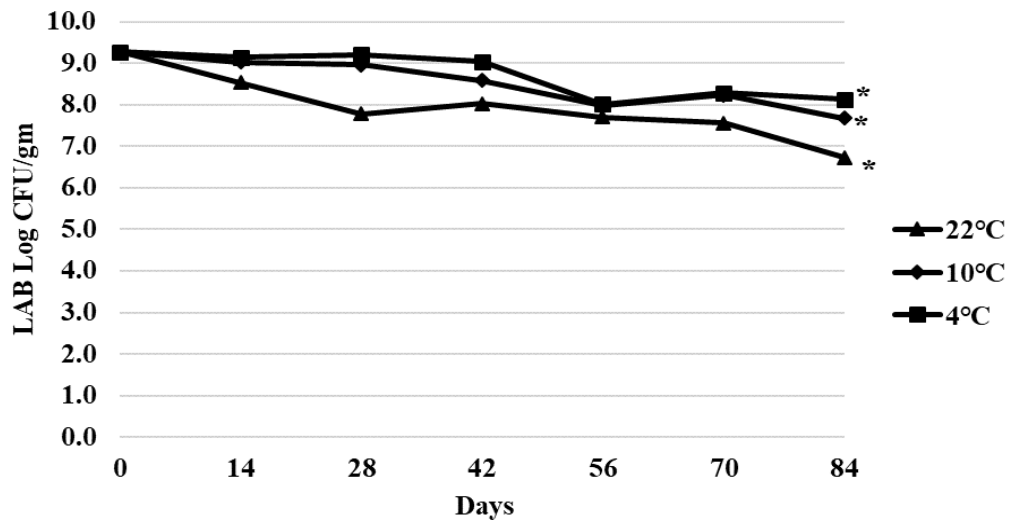


Figure 3-3 The Effect of Temperature on Lactic Acid Bacteria (LAB) counts (A) Control Cheese and (B) Inoculated Cheese samples During the Cheese Aging Process

Data expressed as mean Log CFU/g \pm standard deviation.

*: represents significant differences across treatments.

n=3

3.3.5 The Effects of Aging Temperatures on the pH Levels of Unpasteurized Cheese during Aging

The pH values were measured in control cheese samples, and Figure 3-4 shows the effects of temperature (4°C, 10°C and 22°C) on the mean pH levels over time. Cheese samples started with a mean pH level of 4.80, and pH levels gradually increased in all control samples over time, regardless of aging temperature. However, when aged at 22°C, cheese samples had a significant increase in pH values compared to the other aging temperatures at day 84, reaching a pH of 7.31 compared to pH levels of 5.07 and 5.58 for the cheeses aged at 4°C and 10°C, respectively.

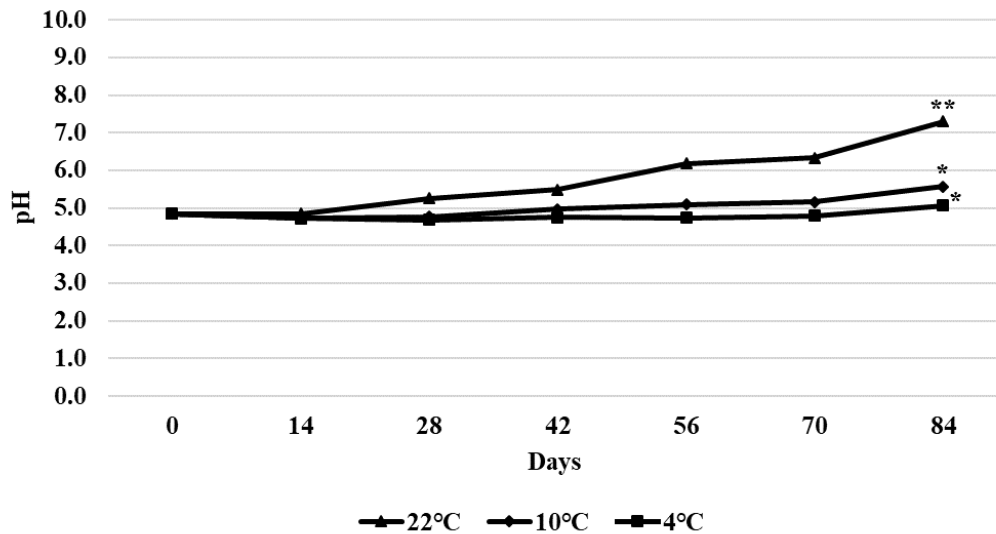


Figure 3-4 The Effects of Temperatures on pH values of Cheese During the Cheese Aging Process.

pH values were expressed as mean \pm standard deviation.

*: represents significant differences across treatments.

n=3.

3.3.6 The Effects of Aging Temperature on the Water Activity Levels and Texture of Unpasteurized Cheese during Aging

The water activity levels were also measured in control cheese samples, and Figure 3-5 shows the effects of aging temperature (4°C, 10°C and 22°C) on mean water activity values. All cheese samples had an average starting water activity level of 0.980. There were no significant ($P > 0.05$) changes in water activity across treatments by the end of aging process.

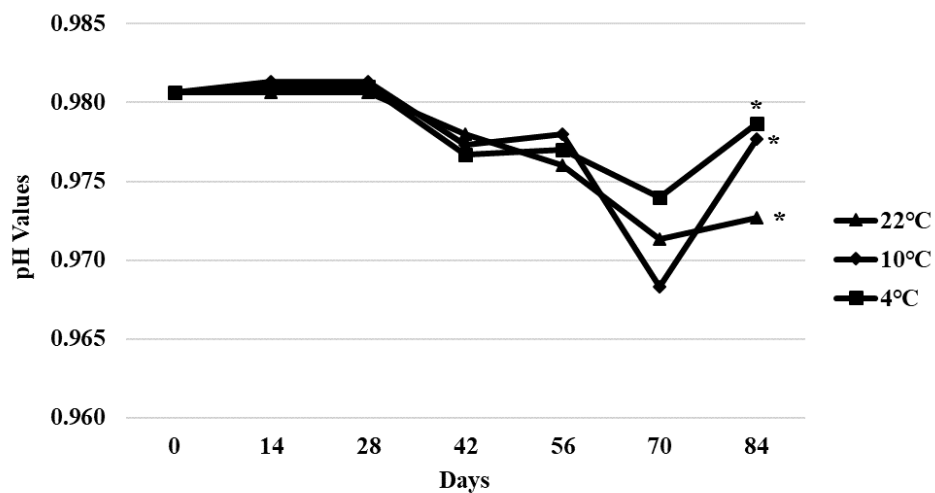


Figure 3-5 The Effect of Temperature on the Water Activity Levels During the Aging Process

Water activity levels were expressed as mean \pm standard deviation.

*: represents significant differences across treatments.

n=3.

The effects of aging temperature (4°C, 10°C and 22°C) on the control cheese texture are illustrated in Table 3-1. Since no texture differences were detected on day 0, the texture was only analyzed on day 84. For cheese samples aged at 4°C, the mean texture values remained stable at day 84. However, aging samples at the higher temperatures (10°C and 22°C) appeared to soften the cheese texture over time, as both samples had significantly ($p < 0.05$) lower compression force values at day 84 compared to 4°C.

Table 3-1 Unpasteurized Cheese Texture Analysis

Aging Day	Temperature °C	¹ Peak Force (g)
0	4	1992.33±384.84a
	10	
	22	
84	4	1815.33±208.74a
	10	641.33±133.37b
	22	244.00±57.29b

¹Peak force (Hardness): represents mean ± standard error.
 Different letters represent significant differences across treatments.
 n=3.

3.3.7 The Effects of Temperature on Fungal Counts in Unpasteurized Cheese After Aging

The control and inoculated cheese samples were plated to enumerate fungal counts after the aging process was complete on day 84 Figure 3-6. There were no significant ($p > 0.05$) temperature effects on fungal populations in the control samples during the aging process. However, aging temperature had a significant ($p < 0.05$) effect on fungal populations in the inoculated cheese samples. Fungal populations were significantly lower at 4°C (6.31 ± 0.25) compared to 10°C (7.62 ± 0.29) by the end of aging period.

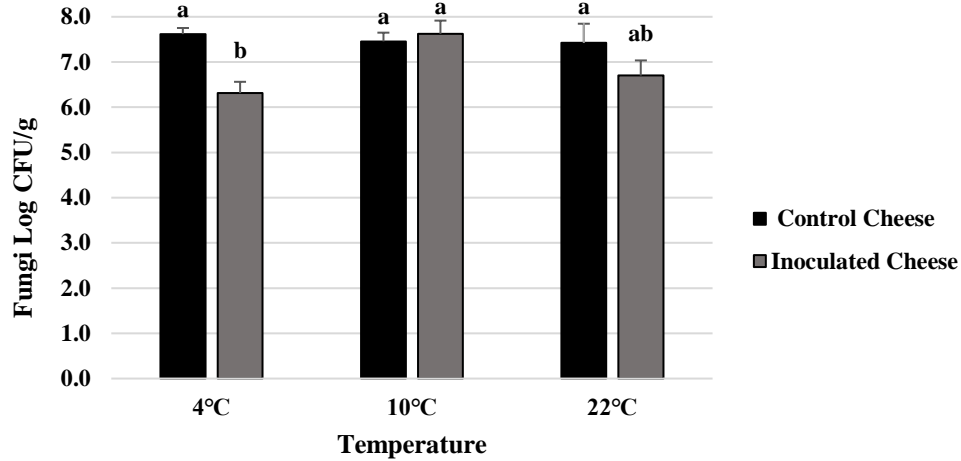


Figure 3-6 The Effect of Temperature on the Fungi Population at the End of the Aging Process

Fungi Log CFU/g were expressed as mean \pm standard error.

Significant ($p < 0.05$) differences between treatments, ANOVA (Tukey's HSD).

n=3.

3.4 DISCUSSION

This study investigates the survival of STEC (O26:H11 and O111:H8) and *L. monocytogenes* (serotype 4b and serotype 1) during the manufacturing and aging of unpasteurized cheese, and the effects of aging temperature (4°C, 10°C, and 22°C) on cheese samples over time. The standard plate count of the fresh cow's milk used for this study was 1,000 CFU/ml, which was within the state legal limit of less than 50,000 CFU/ml⁶¹. The milk samples were plated onto selective media and the results were negative for the targeted pathogens in the control milk. Within the first 24 hours after making the cheese samples, the inoculated pathogens in the samples increased by approximately 1 log CFU/g. This increase can be attributed to the growth of the pathogens in the cheese matrix, which was most likely supported by the composition of the cheese, water activity, and pH levels, and salt content^{12,18,254,255}. In the same manner, other studies also

reported an increase of 1 log in *L. monocytogenes* and *E. coli* O157:H7 counts during the first 24 hours of manufacturing different kinds of cheeses^{12,246,256,257}. In contrast with the findings of this study, Samelis et al. (2009)²⁵⁸ investigated the reduction of *L. monocytogenes* and *L. innocua* counts during the first 24 hours of manufacturing traditional Greek Graviera cheese, which may have been due to an increase in the counts of both mesophilic and thermophilic LAB.

In the inoculated milk, pathogen counts did not change (lag phase) during the initial cheesemaking steps (approximately first two hours). This could be attributed to the adjustment of bacterial cells to a new environment before starting an exponential growth phase²⁵⁹. There are several factors which affect the duration of lag phase, including bacterial inoculum size, the physiological history of bacterial cells, and the physiochemical properties of both the original growth and the new growth environments²⁶⁰. Thus, in this study, the difference in the composition and physiochemical properties of the original medium (TSB supplemented with 0.6% yeast extract) which was used to grow each pathogen in a nutrient rich overnight culture and then transition to milk, could be another potential contributor to the pathogen entry and length of the lag phase. During the cheesemaking process, the bacteria encounters conditions, which may initiate internal stress responses, including the production of antimicrobial agents by the lactic acid bacteria (LAB) metabolic activity, low pH levels due to the production of lactic acid, heat shock and osmotic stress²⁴¹. Thus, STEC and *L. monocytogenes* have developed general tolerance responses²⁴¹. Consequently, bacterial cells enter the lag phase to not only adapt to new environmental conditions before reproduction, but also enable survival within unsuitable mediums^{261,262}. Other studies have also reported no change in *L. monocytogenes* and *E. coli* O157:H7 lag phase growth during the first two hours of the cheesemaking process^{246,257,256}, although they were studying different cheese models than in this study.

The acidification of milk in the cheese manufacturing process may induce an acid tolerance response in these pathogens; therefore, pathogen survival for a longer duration of the cheese ripening process is not surprising assuming that they have counteracted the acidified conditions of the cheese process²⁶³. LAB produce antimicrobial agents during the cheesemaking, including organic acids, bacteriocins, carbon dioxide, hydrogen peroxide, and diacetyl²⁶⁴. The organic acids penetrate the bacterial cytoplasmic membrane of bacteria in the form of protonate, and in this situation both H⁺ and anions can accumulate²⁶⁵. Therefore, the influx of protons and anions can interrupt the pH homeostasis and reduce the internal pH of the bacterial cell. This reduction of the internal pH can denature the pathogenic bacterial enzymes²⁶⁶. The ability of organic acids, such as lactic, acetic and propionic acids, to cause sublethal injury of *E. coli* and *L. monocytogenes* cells by disrupting the outer membranes has been well documented^{267–269}. Consequently, *L. monocytogenes* and *E. coli* can overcome the lethal effect of low pH levels by developing acid tolerance responses (ATR) to mild pH (5.5), including the glutamate decarboxylase-(GAD) system, the arginine diaminase system, and sigma factors^{266,270, 271}. Kroll and Patchett (1992)²⁷² reported a 40-fold increase in *L. monocytogenes* survival after incubating at pH 5.0 compared to the survival of the same pathogen when previously grown at pH 7.0. Applied to this study, the survival of these studied pathogens in the acidic environment of cheese could be attributed to the induction of these responses. The impact of ATR on the survival of pathogens in acidic environments is due to the intracellular decarboxylation in the cytoplasm, which neutralizes the extra protons, and then increases the cytoplasmic pH^{266,273}. Additionally, sigma factors are produced under stress conditions and induce the bacteria to enter the stationary phase and become more resistant and maintain their viability²⁷⁴. Adaptation of foodborne pathogens to mild acidic environments not only improve their survival against lethal pH levels, but also provides cross-

protection against other stresses, including heat shock, osmotic stress, oxidative stress, cold, and bacteriocins^{270,275}.

Other researchers have found that pathogens still survive and reproduce after cutting the curd due to the physical entrapment of bacterial cells in the curd^{20,250}. In this experiment, heat inactivation of both STEC and *L. monocytogenes* was not likely to occur in this farmstead-style cheese replication because the curd was cooked until the temperature reached 39°C, and the duration was a relatively short period of time (approximately 15-20 min.). Our results were in contrast with other studies, since different types of cheeses are made with different methodologies. Ercolini et al. (2005)²⁷⁶ found that *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *Salmonella* spp. counts were reduced in cheese cores due to higher cooking temperatures (55°C). Spano et al. (2003)²⁷⁷ reported that *E. coli* O157:H7 counts were reduced in the curd after stretching it in hot water at 80°C/5 minutes during the manufacturing of unpasteurized Mozzarella cheese. Similarly, Fusco et al. (2012)²⁷⁸ reported *E. coli* O157:H7 counts decreased when the temperature of the curd core was 55°C after stretching the curd for 5-8 minutes in hot water at 90°C.

However, these cheeses received much higher heat temperatures. Subsequently, we observed a significant 1 log reduction in *L. monocytogenes* counts in the whey, compared to counts in the curd during whey expulsion. This decrease could be attributed to several factors, including the sum of stresses during the cheesemaking process, the presence of native microflora, high concentrations of LAB, the lower pH levels in the whey, and possibly an interaction between *E. coli* and *L. monocytogenes* strains^{241,256}. Schleeser et al. (2006)²⁰ also observed a 1 log decrease in *E. coli* O157:H7 counts in the whey during the manufacturing of unpasteurized cheddar cheese.

Exposing cheese to different aging conditions can influence pathogen survival during the aging process. The STEC counts in this study started to decrease after the first week of aging at

the coolest aging temperatures used (4°C and 10°C). A steady reduction in STEC counts was observed until day 56. However, STEC counts were still detected in the enriched samples at both temperatures by the end of this study. A similar trend was observed for the *L. monocytogenes* counts, which started to decrease after the first week of aging at the lowest aging temperature (4°C). *L. monocytogenes* counts slowly decreased until day 63 at this temperature, and after day 63, *L. monocytogenes* cells were detected only in the enriched samples until day 84 of aging at 4°C. Likewise, the counts of LAB slowly decreased in both control and inoculated cheese samples during aging at 4°C. The ability of *L. monocytogenes* and *E. coli* to grow over a wide range of temperatures (2-45°C and 10-46°C), respectively, makes the control of these foodborne pathogens at these temperatures difficult^{279,280}. This ability to survive over a wide temperature range allows these foodborne pathogens to survive at low temperatures and to compete with other microorganisms in the food system^{279,281}. In this study, the survival of the inoculated pathogens at refrigeration temperatures (4°C and 10°C) could be attributed to the induction of cold shock proteins (Csps)^{282,283}. These proteins bind to RNA and DNA and help in the control of replication, transcription, and translation²⁸⁴. Csps, including enzymes, such as denaturase, are associated with modifications of cell membranes in response to temperature²⁸⁵. When the bacteria are exposed to cold stress conditions, the uptake of compatible solutes, such as glycine betaine and carnitine increases, which are involved in the osmotolerance response²⁸⁶. These solutes act as cryoprotectant compounds at low temperatures, which protect the bacterial cells²⁸⁷. Another mechanism that enables both *E. coli* and *L. monocytogenes* to survive at low temperatures is an increased proportion of unsaturated membrane lipids, which helps enhance the fluidity of the membranes²⁸⁸.

Unlike the decrease of STEC counts during aging at 10°C, the counts of *L. monocytogenes* increased consistently over time to 6.4 log CFU/ml up to day 84, which was a significant increase

from 5.7 log CFU/ml at day 0. Aging at the higher temperature (22°C), caused an increase in both STEC and *L. monocytogenes* counts over time. The increase in these pathogen counts were paralleled with a decrease in the LAB counts of both control and inoculated cheese samples, and a gradual increase in pH levels to reach a maximum pH of 5.58 at 10°C and 7.31 at 22°C by day 84. The optimal pH level for *L. monocytogenes* growth is ≥ 4.4 , whereas *E. coli* thrives at pH levels ≥ 4.0 ²⁸⁹.

The decrease in the LAB counts in the control cheese samples were not significantly ($p > 0.05$) different between aging temperatures, whereas aging temperature was a significant ($p < 0.05$) factor in LAB counts among inoculated cheese samples. Specifically, the LAB counts had a one-fold reduction at 22°C compared to 4 and 10°C. This could be attributed to the autolysis of LAB and to the high growth of fungi (approximately 7.5 log CFU/g) in the control samples, which was observed by the end of aging among all temperatures. In the inoculated samples, the LAB counts at 10°C and 22°C decreased progressively, which may be attributed to the same reasons as mentioned above. Autolysis of LAB is caused by releasing the intracellular enzymes into the cheese curd, and the resulting lytic cells provide ripening enzymes, including proteinases, peptidases, lipases, and esterases, which enhance or increase the development of cheese flavor²⁹⁰. Thus, the increase of pH levels in our observations could be attributed to the utilization of lactic acid by molds and to release ammonium through proteolysis²⁹¹. The increase of pH combined with the process of proteolysis, might have inhibited the influence of bacteriocins²⁹², which results in the maintenance of the growth of *L. monocytogenes* during aging at 10°C and the growth of both *L. monocytogenes* and *E. coli* at 22°C.

The water activity within a food matrix is an important intrinsic parameter that can affect microbial growth. In the results of this study, the water activity of control cheese samples did not

appear to be significantly affected by aging temperature (4, 10, and 22°C) over time. The minimum water activity requirement for pathogen growth is 0.95 for *E. coli*²⁹³ and ≥ 0.92 for *L. monocytogenes*²⁹⁴. The water activity values in this study were within the maximum (0.95-0.99) growth rates that are required for many pathogenic bacteria²⁹⁵, and could be another potential reason for the survival of the pathogens examined in this study. The stability of the water activity levels among cheese samples over time was likely due to the vacuum packaging, which prevented water vapor loss during the aging process²⁹⁶.

Previous studies have also investigated the survival of *E. coli* in inoculated aged cheeses at refrigeration temperatures. For example, Reitsma and Henning (1996)¹⁹ investigated the survival of three strains of *E. coli* O157:H7 after 158 days of aging pasteurized cheddar cheese at 6-7°C after vacuum packaging. Similarly, Schleeser et al. (2006)²⁰ reported that *E. coli* O157:H7 survived for 360 days at all initial inoculation levels (10^1 , 10^3 , and 10^5 CFU/ml) in enriched samples of unpasteurized cheddar cheese (pH 5.28) post vacuum packaging and aging at 7°C. D'Amico et al. (2010)¹² also found that three strains of *E. coli* O157: H7 with an initial inoculation level of 20 CFU/ml survived in enriched samples of unpasteurized Gouda (pH 5.16) and cheddar (pH 5.20) cheeses after aging for 270 days at 9°C. In other acidified fermented foods, such as fermented sausage, Glass et al. (1992)²⁹⁷ reported that *E. coli* O157:H7 survived for eight weeks at a pH level of 4.8 and stored at 4°C. In contrast to the inoculation method that was used in this study, other studies have used surface inoculation methods. D'Amico et al. (2008)¹⁸ found that a cocktail of five strains of *L. monocytogenes*, when inoculated at a low initial level of 20 CFU/ml, increased after 60 days of aging on pasteurized and unpasteurized surface mold-ripened soft cheese at 4°C. Valero et al (2014)²⁹⁸ documented that the initial inoculation level of 4 log CFU/g of *L. monocytogenes* decreased after 60 days of aging in unpasteurized sheep's milk cheese vacuum

packaged and stored at 4°C, 12°C, and 22°C, and *L. monocytogenes* survived until day 114, 104, and 77 days, respectively. The researchers attributed the faster reduction of *L. monocytogenes* counts in the cheese aged at 22°C to the loss of humidity and the excess production of metabolites by LAB that inhibited their growth. Shrestha et al (2011)²⁹⁹ reported that *L. monocytogenes* counts reduced gradually in a post-aging contamination study in pasteurized cheddar cheese after aging for 30 days at 21°C. Post-processing contamination of cheese with foodborne pathogens during ripening, slicing, and storage can potentially occur³⁰⁰.

In addition to the effects of aging temperatures on pathogen survival, LAB counts, pH and water activity levels; the texture profile analysis (TPA) of control cheese samples was analyzed on day 0 and 84 of aging at 4, 10, and 22°C. Aging at a low refrigeration temperature (4°C) maintained the firmness of the cheese texture over time. One potential reason could be the slow occurrence of proteolysis at this temperature, which could be attributed to the high moisture content and relatively low pH level. After 60 days of aging at 10°C and 22°C, a decrease in cheese firmness in samples was observed. This could be due primarily to a higher rate of proteolysis at these aging temperatures²⁹¹. Awad (2006)³⁰¹ reported the hardness of Ras cheese (a popular hard cheese in Egypt made from a mixture of unpasteurized cow and buffalo's milk) had decreased by the end of aging at 13±2°C. The authors found that this was due to the higher rate of proteolysis. During cheese ripening, several factors, including pH, salt, and moisture content influence the cheese texture³⁰². Proteolysis is considered an important process during cheese ripening, which affects both the cheese flavor and the cheese texture³⁰³. During cheese ripening, proteolysis is catalyzed by several sources of enzymes including rennet, milk plasmin and cathepsin D, enzymes from starter cultures and non-starter cultures, and lastly from added secondary cultures, such as *Penicillium roqueforti*¹⁹⁰. In the initial stages of cheese ripening, rennet hydrolyzes the Ph₂₃-Ph₂₄

bond of α_{s1} -casein, resulting in large peptides (water-insoluble) and intermediate-sized peptides (water-soluble), which causes the initial softening of the cheese texture³⁰⁴. After this preliminary softening, these peptides are further broken down into shorter peptide chains due to rennet and other enzymes present such as the extracellular proteinases from the starter culture. Consequently, peptidases hydrolyze these short peptides which further promotes the formation of free amino acids¹⁹⁰. Changes in the cheese texture are attributed to the breakdown of the protein network, cause a decrease in water activity through water binding by liberated carboxyl and amino groups, as well as an increase in pH cheese levels¹⁹⁰. Thus, as is observed in this study, a gradual increase in pH and gradual decrease in water activity levels during aging at both 10°C and 22°C might have accelerated proteolysis, which resulted in the softening of the cheese texture over time.

3.5 CONCLUSIONS

Overall, it is difficult to compare our observations with previous studies due to the many variables applied during the cheesemaking and aging process, in this study such as the initial inoculum level, pathogens serotypes used, starter culture behavior, aging temperatures and use of vacuum packaging that can all influence pathogen survival in different ways. The initial inoculum level used in this study was above the level of contamination that would realistically be found in cheeses produced under good manufacturing and sanitation practices, which allows for clearer observation of population dynamics. However, even when realistically low inoculum levels were used in other studies, such as D'Amico et al. (2010)¹², similar survival trends were observed. Both *E. coli* and *L. monocytogenes* are two of the most common foodborne pathogens associated with ready to eat cheese products. A combination of natural antimicrobial agents in the milk, antimicrobial metabolites from the starter cultures, low pH levels, and aging temperatures are potential factors that may affect the survival of foodborne pathogens in dairy products.

Cheesemakers may also use higher cooking temperatures for certain types of cheese, which may effectively reduce pathogen populations within the milk. In agreement with previous studies, the policy of the FDA regarding 60 days of holding unpasteurized milk cheese should be reconsidered. We can conclude that aging at the lowest temperature possible will provide the best control against both Shigatoxigenic *E. coli*, and most importantly *L. monocytogenes*, and can be utilized by small-scale cheese makers to ensure the safety of their products.

3.6 LIMITATIONS

The two major limitations of this study were vacuum packaging and cheese sample size. Vacuum packaging was used to prevent cross contamination with lab equipment and personnel within the aging space since the treatment cheese samples were experimentally inoculated with the pathogens of interest. This process might have maintained the survival of pathogens, and may also have excluded the growth of other microbes during the aging conditions in our observations. In this study, the cheese samples were cut into small pieces ($\sim 1 \text{ in}^3$) in order to avoid complexity introduced by a differential pathogen surviving across different regions of a large cheese sample.

CHAPTER 4 DETECTION OF *LISTERIA* SPP. IN UNPASTEURIZED RETAIL DIARY PRODUCTS IN MAINE

4.1 INTRODUCTION

The Maine dairy industry is a significant contributor to the state's economy¹. Maine is among thirty states that legally permit intrastate sale of unpasteurized dairy products in both direct-to-consumer and retail sales³⁰⁵. Consumer demand has increased for unpasteurized fluid milk and milk products, most notably in Maine. However, with this increased demand comes increased risk of foodborne illness as consumption of contaminated, unpasteurized fluid milk and milk products in the U.S. has been well reported^{129,198,199}. Results from previous investigations have clearly shown that *Campylobacter*, *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* are among the most common foodborne pathogens to cause foodborne diseases within this product type^{129,306}.

Dairy farms are ideal harborage sites for various microorganisms including *Listeria* spp.^{66,222}. *Listeria* is ubiquitous in the environment including soil, water, grass, silage, sewage, and animal feces^{87,307}. *Listeria* spp. are Gram-positive, motile, intracellular, facultative anaerobes, which can survive in a wide range of pH (4.0-9.5), temperatures (1-45°C) and water activity \geq 0.92. In addition, these species are capable of growth in 10% w/v of NaCl³⁰⁸. There are 17 recognized species in the *Listeria* genus; however, only *Listeria monocytogenes* and *Listeria ivanovii* are considered pathogenic for humans and animals, respectively³⁰⁹. It is very well-known that *L. monocytogenes* can contaminate raw milk from the dairy farm environment³¹⁰. Skovgaard and Morgen (1988)³⁰⁷ reported there was a relationship between silage quality and the presence of *Listeria* spp. in feces. The risk of fecal contamination of raw milk by *Listeria* spp. can occur during

seasons when the animals housed indoors due to limited space between animals and silage consumption³¹¹.

L. monocytogenes is most frequently implicated in foodborne illness outbreaks; however, other *Listeria* species isolated within these environments can serve as indicator organisms for this pathogen³¹². Therefore, the recovery of any *Listeria* species from a processing environment is cause for concern because of the small infectious dose and high mortality rate associated with listeriosis infection⁹⁸. For this reason, the FDA has implemented a zero-tolerance policy for this microorganism in foods, and is especially a concern for ready-to eat-foods¹⁴².

Coliforms are a broad classification of microorganisms, which are indicators for possible fecal contamination. Genera comprising this grouping include *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*^{66,313}. Coliform bacteria are widely found in fluid milk³¹⁴, in many cheeses⁶³, and other dairy products. These bacteria can originate from different sources in the dairy farm environment such as water, equipment, plant materials, and fecal sources³¹⁵. Cheese characteristics, including water activity and pH levels, salt content, aging conditions, and starter cultures, all influence the possible microbial profile in the final product³¹⁶. Coliform bacteria are natural inhabitants of the gastrointestinal tract of warm-blooded animals. These bacteria are characterized as Gram-negative, aerobes or facultative anaerobes and non-spore formers. An additional defining feature of this group is that they are able to ferment lactose and produce acid within 48 hours at 35°C³¹⁷. Fecal coliforms, including *E. coli*, *K. pneumoniae*, *C. freundii*, and *Enterobacter* spp., are a subgroup of coliform bacteria which can grow at 44°C and ferment lactose³¹⁸. Producers will test for coliforms as an indication of overall facility sanitation and cleanliness. The FDA defines the maximum coliform levels in the Pasteurized Milk Ordinance (PMO) as less than 10 CFU/ml⁵⁸. Although there is no universally accepted threshold for coliform

presence in unpasteurized milk products, states which permit the sale of this product type have typically enforced ranges below 100 CFU (ml or g). Maine enforces the more rigorous end of this specification citing no more than 10 CFU/ml³¹⁹.

Generally, fresh milk drawn from healthy cows is usually free from pathogens; however, milk becomes exposed to potentially pathogenic bacteria once a cow is milked. Several sources, including unhygienic milking equipment, milkers' hands, feces, silage, contaminated water and grass, are the cause of this contamination³¹¹. Furthermore, *Listeria* contamination of raw milk cheeses are well reported. Past studies have documented the prevalence of *L. monocytogenes* and *Listeria* spp. in different types of cheese including soft, semi soft, and mold-ripened cheeses^{320–322}.

Even though prior studies haven't reported a correlation between coliforms as indicators of a broader range of potential pathogens in dairy products, it may be possible due to the manufacturing practices of these product types^{18,323,324}. Therefore, the primary objectives of this study were to possibly bridge the gap within this potential correlation between pathogens and coliforms by (1) to determine if there may be a potential correlation between *Listeria* spp. and coliforms in unpasteurized dairy products, and (2) to investigate the prevalence of *Listeria* spp. in different types of Maine unpasteurized cheese.

4.2 MATERIALS AND METHODS

4.2.1 Samples

One hundred and four unpasteurized retail dairy products, including 45 cheeses and 59 milk samples, were collected weekly from the Milk Quality Laboratory at the Maine Department of Agriculture, Conservation and Forestry in Augusta, Maine from June through October of 2018. Samples were collected from 28 consenting dairy farms throughout the state of Maine that agreed

to participate in this study. Milk samples were collected in sterile plastic containers while cheese products were packaged into individual sterile polyethylene bags. After collection, the samples were transported in a cooler under refrigeration. The studied cheese samples were categorized into soft and mold-ripened cheeses because these were the cheese types that were more frequently produced by participating dairy producers in this study.

4.2.2 Microbiological Analysis

Coliform data were generated by dilution plating of retail samples onto 3M™ Petrifilm™ Coliform Count Plates in the Milk Quality Laboratory at the Maine Department of Agriculture, Conservation and Forestry in Augusta, Maine. All samples were collected aseptically by state inspectors and analyzed by trained laboratory technicians. The results were reported in colony forming unit (CFU) per ml or g with greater than 10 CFU/ml or g being non-compliant with state regulations.

Detection of presumptive *Listeria* spp. in unpasteurized dairy samples was conducted at the Pathogenic Microbiology Laboratory at the School of Food and Agriculture at the University of Maine, Orono, Maine. Buffered Listeria Enrichment Broth (Alpha Bioscience, Baltimore, MD, USA) was used to enrich *Listeria* spp. Each 25 ml sample of unpasteurized retail milk was aseptically placed into a sterile polyethylene bag with 225 ml of 1X of the enrichment broth. Each 25 g cheese sample was placed into a sterile blender, diluted in warm (45°C), sterile 2% (w/vol) sodium citrate dihydrate (Fisher Chemical, Fair Lawn, NJ, US). Samples were then homogenized for 2-3 minutes using a sterile Waring Commercial Blender 700S (Torrington, Connecticut, USA), and then a solution of 2X selective enrichment medium was added. Enrichments were incubated at 30±1°C for 24 hours. After incubation, aliquots of enriched samples were plated onto modified

Oxford agar (MOX; Alpha Biosciences, Baltimore, MD, USA). Preparation of this media requires the addition of modified oxford antibiotic supplement (BD Difco; Sparks, MD, USA) (FDA BAM, 2017). Plates were again incubated at $30\pm 1^{\circ}\text{C}$ for 24 hours before examination for colonies with typical morphology.

4.2.3 Statistical Analysis

Chi-Square tests of independence were used for comparisons of categorical variables. Pearson Chi-Square was used to determine the association between coliforms and *Listeria* spp. therein, other variables (months and products) were observed using the Pearson correlation coefficient at significance levels $p < 0.01$ and $p < 0.05$. Mean temperature differences were determined by using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) for mean separation. P values < 0.05 were considered statistically significant and data were expressed as mean \pm standard errors. The analyses were performed by using IBM SPSS Statistics version 25 (Orem, UT).

4.3 RESULTS

4.3.1 The Correlation between *Listeria* spp. and Coliforms in Unpasteurized Retail Dairy Products.

Table 4-1 shows the correlation between *Listeria* spp. and coliforms. The overwhelming majority of samples compliant for coliforms tested negative for *Listeria* spp. When samples were non-compliant for coliforms, there were almost the same chance of getting presumptive positive and negative for *Listeria* spp. The Pearson's correlation showed there was a significant ($p < 0.05$) association between *Listeria* spp. and coliforms (Pearson Chi-Square = 20.099, $p = 0.000$).

Table 4-1 Pearson Correlation Between Coliforms and *Listeria* spp. in Unpasteurized Maine Dairy Products

	* ($p = 0.000$)	
	% <i>Listeria</i> spp.	
Coliforms	Positive	Negative
Compliant	6.9	93.1
Non-Compliant	43.8	56.3

*Pearson Chi-Square between the Presence of *Listeria* spp. and Non-compliant Coliforms is significant at level (0.05).

4.3.2 The Prevalence of *Listeria* spp. and Coliforms in Different Types of Unpasteurized Retail Cheese.

The prevalence of *Listeria* spp. in unpasteurized retail cheeses is displayed in Table 4- 2. The results showed that 88% (22 of 25) of soft cheese and 77.4% (14 of 18) of mold-ripened cheese samples were *Listeria* spp. negative. The presence of *Listeria* spp. was not significantly ($p > 0.05$) correlated with the cheese types as shown in Table 4- 2.

Table 4- 2 Prevalence of *Listeria* spp. in Different Types of Unpasteurized Retail Cheese

	$p = 0.370^*$	
	% <i>Listeria</i> spp.	
Cheese Types	Negative	Positive
Soft	88.0	12.0
Mold-Ripened	77.8	22.2

*Pearson Chi-Square between the Presence of *Listeria* spp. and Chees Types is significant at level (0.05). Soft Cheese n= 25, Mold-Ripened Cheese n= 18.

4.3.3 The Effect of Months on the Prevalence of *Listeria* spp. and Coliforms in Unpasteurized Retail Dairy Samples.

Table 4- 3 displays the effects of months on the occurrence of *Listeria* spp. and coliforms in the studied samples. No trend was observed between month and the occurrence of non-compliant coliform levels throughout the study. The highest percentages of non-compliant coliform levels were observed in July, October, and September. The occurrence of presumptive *Listeria* spp. was significantly ($p < 0.05$) higher in July and declined in August as shown in Table 4- 3. The prevalence of *Listeria* spp. was significantly ($p < 0.05$) correlated with higher temperatures, whereas non-compliant coliforms was not as shown in Table 4- 3.

Table 4- 3 Prevalence of *Listeria* spp. and Coliforms during the Study in Unpasteurized Maine Dairy Products

	* ($p = 0.143$)		* ($p = 0.000$)	
	% Coliforms		% <i>Listeria</i> spp.	
Months	Compliant	Non-compliant	Negative	Positive
June	80.0	20.0	100	0.0
July	55.0	45.0	45.0	55.0
August	82.2	17.2	75.9	24.1
September	71.4	28.6	100	0.0
October	57.7	42.3	96.2	3.8

*Pearson Chi-Square between the Prevalence of Non-compliant Coliforms and *Listeria* spp. is significant at level (0.05).

June n= 15, July n= 20, August n= 29, September n= 14, October n= 26.

Figure 4-1 shows the variation of mean temperatures among months. Temperatures in July ($22.03^{\circ}\text{C}\pm 2.47$) and August ($21.94^{\circ}\text{C}\pm 2.78$) were significantly ($p < 0.05$) higher than temperatures in June (17.27 ± 3.72), September ($16.80^{\circ}\text{C}\pm 4.36$), and October ($7.77^{\circ}\text{C}\pm 4.43$). The mean

temperature in October was significantly ($p < 0.05$) lower than the mean temperatures of the other months.

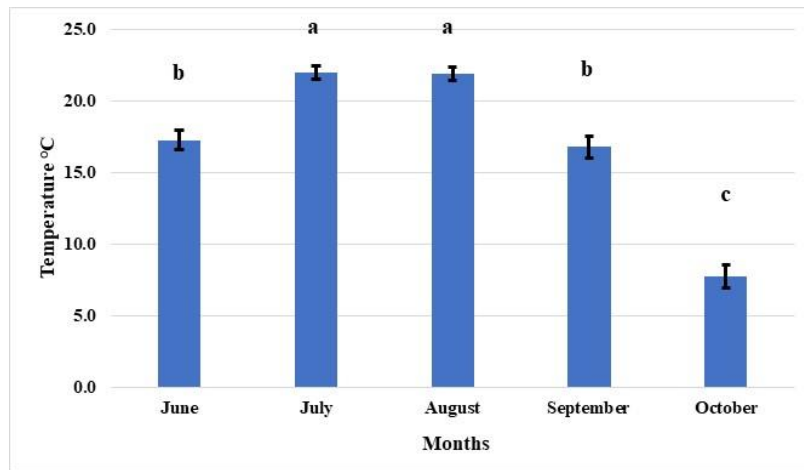


Figure 4-1 The Trend of Maine's Temperatures Across Study Duration

Temperatures were expressed as mean \pm standard error.

ANOVA was used and significant differences between means were analyzed using Tukey's HSD. The columns not sharing the same letters are significantly different ($p < 0.05$) across months.

4.4 DISCUSSION

The State monitoring procedures for pathogen detection in unpasteurized retail dairy products have not been established within the state of Maine. This study, however, investigates the correlation between *Listeria* spp. and coliform counts in these products and detects the occurrence of *Listeria* spp. in soft unpasteurized retail cheese samples. Based on our results in Table 4-1, we found that there was a significant ($p < 0.05$) correlation between the prevalence of presumptive positive *Listeria* spp. and non-compliant coliform counts. The study also showed that samples with compliant coliform levels were negative for *Listeria* spp. in over 93% of cases, which

may indicate that low coliform levels are a fairly reliable indicator for the absence of *Listeria* species. It should be noted that both coliforms and *Listeria* spp. are environmental microorganisms that can be found on dairy farms, which might result in the possible contamination of the milk and/or dairy products. On the other hand, the results suggest that non-compliant coliform levels are correlated with an increased risk of *Listeria* spp. presence, but cannot be considered a plausible indicator of non-compliant samples for coliforms, since these samples had nearly equal percentages of presumptive positive (43.8%) and negative (56.3%) results for *Listeria* spp. Other studies have also investigated the correlation between coliform bacteria and the presence of foodborne pathogens in milk samples. The overall conclusions from their results were correlation was not significant, and coliform bacteria cannot be used as an index organism for any pathogens because these microorganisms can be naturally found in the farm environment. In addition, the highest quality unpasteurized milk products cannot guarantee pathogen absence^{11,18, 89,325,326,316}.

Listeriosis can cause severe health implications in cow herds; however, shedding the organisms in feces from subclinical animals can also occur^{327,328}. Furthermore, feeding practices, such as the use of poor-quality silage, can also be a source of pathogenic bacteria³²⁹. Silage is produced through anaerobic fermentation of a moist forage crop by the acidification process of carbohydrates after being converted into organic acids through the action of lactic acid bacteria (LAB)³³⁰. Lactic acid bacteria's ability to reduce pH in this feeding material extends the product's shelf life while minimizing nutrient losses. Additionally, this ability inhibits the growth of unwanted epiphytic bacteria in plants, including *Clostridium* spp., *Bacillus* spp., coliform bacteria, yeasts and molds by producing antimicrobial agents^{330,331}. The occurrence of pathogenic bacteria in silage is attributed to contamination or improper fermentation processes, which favor the growth of pathogens³³². *Listeria* spp. including *L. monocytogenes*, have been isolated from poorly

fermented silage with pH levels > 4.0 ^{333,334}. Thus, the presence of *Listeria* spp. in the milk could be attributed to the transmission of these organisms from contaminated silage into the fluid milk.

L. monocytogenes is considered a severe threat to the dairy industry. In general, unpasteurized dairy products are more vulnerable to pathogens due to the lack of heat processing. The occurrence of *Listeria* spp. in unpasteurized cheese products is mainly from contaminated milk or post-processing contamination. High mortality rates from human listeriosis have led to the FDA to establish a zero-tolerance policy in RTE foods at any detectable level³³⁵. Thus, the detection of foodborne pathogens is crucial to ensure the safety of unpasteurized dairy products. The potential presence of *Listeria* spp. in two types of unpasteurized milk cheeses was detected in this study. The results showed that both soft and mold-ripened cheeses had tested presumptive positive for *Listeria* spp.; however, mold-ripened cheeses had almost doubled the percentage (22%) of presumptive positives for *Listeria* spp. compared to soft cheeses (12%). The incidence of presumptive positives for *Listeria* spp. in the tested cheeses could be attributed to the cheese characteristics and high tolerance properties of *Listeria*. Soft cheeses have food preservative characteristics such as pH levels of 4.5 to 6.5 and a final salt content of 2.3% to 3.5%³³⁶; however, *Listeria* spp. can survive and may induce tolerance responses under pH levels 4.0-9.5 and a NaCl content of 10% w/v^{263,308}. Mold-ripened cheeses may have favorable pH levels for *Listeria* growth. These are typically characterized by *Penicillium camemberti* and *Penicillium roqueforti* growth during ripening. These molds metabolize lactic acid that is produced by the starter cultures to produce ammonia from the amino acids, which elevates the initial pH (4.6) to neutral 7.0³³⁷. This pH value could be the reason that mold-ripened cheeses had higher percentages of presumptive *Listeria* spp. positives in this study. The prevalence of *Listeria* spp. positives namely *L. monocytogenes*, in soft, semi-soft and mold-ripened cheeses have been investigated in other

studies. Loncarevic et al. (1995)³²⁰ in Sweden reported that 42% of unpasteurized retail soft and semi-soft cheeses were positive for *L. monocytogenes*. Other investigations have reported prevalence range of 0.5%-15% of *L. monocytogenes* in soft and semi-soft cheeses made from unpasteurized and pasteurized milk³³⁸. Trmcic et al. (2016)³¹⁶ attributed the prevalence of *Listeria* spp. to the cheese characteristics (pH 5.25-7.02 and of water activity levels of 0.939-0.981) of unpasteurized mold-ripened and soft cheeses.

We further observed the effects of the monthly temperatures from June to October on the prevalence of presumptive *Listeria*. A higher percentage of presumptive positive *Listeria* spp. were detected in the summer months (July and August) than in fall (September and October). A study by Gaya et al. (1996)³³⁹ in Spain reported that the contamination samples with *Listeria* spp. from various containers of milk was seasonal but contradicts the seasonality that we observed. The researchers found that the occurrence of *Listeria* spp. was higher in fall and winter than in spring and summer. Another investigation by Abou-Eleinin et al. (2000)³⁴⁰ documented that 35 out of 450 samples tested positive for *Listeria* spp. positive and their prevalence was higher in winter and spring rather than in fall and summer months. Researchers likely detected more *Listeria* spp. in winter and spring compared to summer because outdoor feeding areas where goats graze increases direct animal contact with soil matter and germinating seeds, which are harborage sites for the pathogen. These materials are then routinely ingested by the goat and excreted in the feces. *Listeria* is a psychrotrophic bacterium that can grow at low temperatures³⁴¹. Unlike the previously mentioned study, Ho et al. (2007)¹⁰⁰ found there were no seasonal effects on the prevalence of *L. monocytogenes* and *Listeria* spp. isolated from dairy equipment, which suggests that milk itself is the more likely source of contamination.

It is necessary to mention that in this study all presumptive positive samples were independent of commodity type and producer during summer season with the exception of one dairy producer who provided both a presumptive positive cheese and milk sample. This indicates that the dairy producers had more environmental challenges (temperature, humidity, pest management) in summer months to keep up with proper sanitation practices. There were twelve out of one hundred and four unpasteurized dairy samples that were presumptively positive for *Listeria* spp. with majority belongs to unpasteurized milk samples. This indicates that there are sanitation issues on those farms, which may potentially increase the risk for *Listeria* presence in the finished product.

The current literature demonstrates varied information regarding the validity and accuracy of using specific selective media to detect for *Listeria* spp., and there are some microbes that could cause some false positive results, such as *Enterococcus* species. Thus, the presumptive positive results may vary based on the type of food product in which the pathogen is isolated, as well as the enrichment and detection methods. For example, in this study, there twelve out of a hundred and four (11.53%) of unpasteurized dairy samples indicated positive for *Listeria* spp. after a 24 hours enrichment in BLE broth at 30°C and subsequent plating on MOX for 48 hours at 30±1°C. In a study by Benhalima et al. (2019)³⁴², nine of the forty-two unpasteurized milk samples were positive for *Listeria* spp. after enrichment in two broths (Fraser half concentration enrichment broth and Fraser broth, and then plated on two selective media (MOX and PALCAM). The percentage of false positives detected on each selective agar was 4.76% and 2.22%, respectively. This could be attributed to the type of enrichment broth and the additional biochemical tests used to confirm the *Listeria* genus.

We found there were significant ($p < 0.05$) differences in the incidence of presumptive *Listeria* between months, but the occurrence of coliforms was not significantly ($p > 0.05$) correlated by month. Coliform bacteria form part of the intestinal microflora of animals; thus, their presence in dairy farms is to be expected^{66,313}, and may indicate possible fecal contamination or inadequate sanitation practice and/or hygiene. In our observations, samples with non-compliant coliform levels did not reveal a clear trend in their presence among months. Although the optimum temperature for coliforms is $35 \pm 1^\circ\text{C}$ ³⁴³, other bacteria genera recently termed “environmental coliforms”, including *Serratia*, *Hafnia*, *Rahnella*, *Buttiauxella*, and *Leclercia* have been shown to grow in milk at refrigeration temperatures³⁴⁴. This could explain the prevalence of coliforms in the tested samples that were collected (with average air temperatures of 7.77°C in October and 22.03°C in July). A study by Harmon (1994)²²⁵ reported that during summer months the number of environmental bacteria, such as coliforms in bedding material, increased which could be attributed to favorable temperature and humidity, and is consistent with our findings. Coliforms are a consistent component to the microbial population in unpasteurized milk^{20,217,325,435}. High counts of coliform bacteria in the milk also could be attributed to undiagnosed mastitis in the milking animals²²³. An investigation by Van Kessel et al. (2004)³⁴⁵ documented that 96% of tested bulk tank milk samples in the U.S. in 2002 tested coliform positive. The reported coliform levels in unpasteurized milk in the U.S. were between 31 CFU/ml and 2570 CFU/ml^{208,219,325}.

4.5 CONCLUSION

The findings of this study and other studies suggest that the level of coliform bacteria is not a reliable indicator microorganism to predict the presence or absence of foodborne pathogens, including *Listeria* spp. in unpasteurized dairy products. However, overall low coliform levels seemed in our samples to be correlated to a lower risk of *Listeria* spp. presence. Current detection

methodologies utilize enrichment procedures which may favor or suppress certain *Listeria* spp. Also, the outgrowth of the background flora in raw milk, such as *Enterococcus* species, may give a false positive result for the presence of *Listeria* spp. on some selective media³⁴⁶. Therefore, additional research on alternative detection methods are necessary to reduce the instance of false positives. Similarly, coliforms are ubiquitous in farming environments and therefore, more targeted testing approaches, which specifically test for *E. coli* presence, may be better indicators of fecal contamination sources. Proper farming practices, in addition to routine quality testing, are necessary to improve the ability to detect bacteria of public health concern. Further studies are needed to verify the significant correlation that we found, as well as test samples during winter and spring months and rapid screening pathogen testing is highly recommended.

4.6 LIMITATIONS

This study; however, was subject to several limitations. Limited financial support for this research made investigation into additional detection techniques for *Listeria* spp. not possible. Additionally, a lack of study participants made the sampling size for this work relatively small, thus the data that was generated may be less representative of the true food safety risk associated within these commodities. Lastly, the lack of previous studies in this research area has also limited the comparison to our findings with others.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

To our knowledge, there have been no prior studies which have evaluated the quality and safety of unpasteurized retail dairy products in Maine or investigated the effects of various aging temperatures on pathogen survival used in unpasteurized cheese manufacturing.

The results indicate that a clear trend was revealed between bacterial populations and the time period between 1998 and 2016. However, it should be noted that in both cow and goat milk samples, the highest mean values standard plate and coliform counts were documented in earlier years, whereas the lowest means were recorded more recently. Furthermore, there were no significant differences in the cleanliness status of either cow or goat's milk products, although coliforms were the source of higher non-compliant in the studied samples. However, the variations in the environmental conditions may have had significant effects on bacterial populations, since higher coliform bacterial populations were observed in the summer months compared to winter. Additionally, the results also reveal that low coliform counts appeared to be correlated with a lower risk of presumptive *Listeria* spp., and the cheese type and the environmental conditions had an impact on the prevalence of *Listeria* spp. in the tested unpasteurized retail dairy products.

This research has also clearly shown the growth trends of the studied foodborne pathogens during the manufacturing and aging process of unpasteurized cow's cheese at different aging temperatures. This suggests that small-scale cheesemakers in Maine should use the lowest temperature (4°C) studied during aging in order to control the growth of pathogens in the finished product without altering the level of beneficial lactic acid bacteria or desirable finished product attributes.

We can conclude that Maine's retail dairy market may represent a valuable sector within the thirty states that allow the sale of unpasteurized dairy products. The number of retail

unpasteurized milk samples has had a five-fold increase from 1998 to 2016, which suggests that the market for these types of products continues to expand. Therefore, high quality and more importantly the safety of unpasteurized dairy products is crucial. Additional state monitoring procedures for pathogen detection is highly recommended to better ensure the safety of these products. Further studies are also needed to identify other farm risk factors associated with coliform counts in unpasteurized milk and dairy products.

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