The association between bulk tank milk analysis for raw milk quality and on-farm management practices

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Abstract

The primary objective of this thesis was to investigate the association between raw milk bacterial quality and on-farm management practices in Prince Edward Island dairy herds. Secondary objectives included identification of lipolytic and proteolytic bacteria in pasteurized milk and investigation of the role of mastitis associated pathogens in elevated bacterial counts in bulk tank milk (BTM).

To achieve the primary objective, 4 studies were conducted. In the first study, BTM quality was evaluated biweekly in all PEI dairy herds over a two year period (March 2005 to March 2007), using total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC), coliform (CC), and somatic cell (SCC) counts. The results of this study showed weak correlations among the bacterial counts which suggests that each count gives different information in relation to management practices. With the exception of SCC, other milk quality parameters had moderate to high coefficient of variation, which indicates that herd assessments should not rely on a single measurement. In general, there was no consistent seasonal pattern over the 2 year study period for TAC, PIC, and LPC, although all counts tended to be low in winter. The CC and SCC were always highest in summer.

In the second study, the association between laboratory test results and on-farm management practices was assessed using data from a mail out survey. The survey covered 4 main areas: general farm demographics and management, cow cleanliness and hygiene, milking procedures and mastitis control, and equipment maintenance and cleaning. The response rate of the survey was 65%. The TAC and PIC were positively associated with the amount of soiling on the teats prior to udder preparation and manual cleaning of the bulk tank. Additionally, various methods of premilking udder preparation were important, with pre-dip followed by drying being superior to other methods in reducing the bacterial counts. The LPC was positively associated with the presence of a plate cooler and inadequate frequency of acid washing, whereas having a water purification system was protective. Finally, for CC, clipping udder hair and automated washing of the bulk tank were protective, whereas increasing herd size and inadequate frequency of acid washing were risk factors.

In the third study, the association between BTM bacterial quality and management practices was further investigated using a case control study (January 2006 to May 2007). Cases and controls were defined based on the results of all bacterial counts. On-farm evaluation included observation of basic management practices, evaluation of equipment hygiene and cooling efficiency, and scoring of cow and environmental hygiene. The results identified udder hygiene, milking system wash solution temperature and chemistry, and milk house water quality as important factors for bacteriological quality of BTM.

In the forth study, four case-control groups were evaluated to determine specific on-farm risks for each of TAC, PIC, LPC, and CC. The results of this analysis showed

that TAC and PIC were mainly associated with cow and stall hygiene, washing the teats with water and not using teat pre-dip, and having dirty teats after udder preparation. The LPC and CC were related to equipment hygiene, with high counts being associated with low temperature of the cleaning solution, high water hardness score, and high alkalinity of the alkaline detergent wash.

One of the secondary objectives was to characterize lipolytic and proteolytic bacteria in pasteurized milk. In this study, BTM from 100 farms was subjected to laboratory pasteurization. The lipolytic and proteolytic activity of the surviving bacteria was determined under conditions that approximate poor refrigeration. The predominant isolates from pasteurized milk were Gram-positive rods (83% mainly *Bacillus* spp.), followed by Gram-positive cocci (17% mainly *Staphylococcus* spp.). Most of the isolates showed proteolytic or lipolytic activity or both, which indicate their potential of causing spoilage of pasteurized milk.

Another secondary objective was to investigate the role of mastitis-associated pathogens in elevated TAC in BTM. In 17 (19%) out of 89 samples that had high (>10,000 cfu/ml) TAC, mastitis-associated pathogens had a significant proportional contribution (≥0.25) to the TAC. While the majority of high bacterial counts were not associated with mastitis organisms, mastitis-associated pathogens can be present in very high numbers in bulk tank milk and can contribute to elevated TAC.

In conclusion, TAC, PIC, LPC, and CC are of considerable value for identifying practices that could influence milk quality. Lipolytic and proteolytic bacteria which survive pasteurization, particularly *Bacillus* spp., represent a big challenge to the shelf life of pasteurized milk. Although not common, mastitis associated pathogens could be associated with elevated TAC in BTM.

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List of Abbreviations

BP	Baird Parker
BTM	Bulk tank milk
BTSCC	Bulk tank somatic cell count
CAMP	Christie Atkins Munch-Petersen
CC	Coliform count
CFU	Colony forming unit
CV	Coefficient of variation
EMCO	Edward's Modified agar supplemented with Colistin sulfate and Oxolinic acid
GEE	Generalized estimating equations
GPG	Grain per gallon
ICC	Intra-class correlation coefficient
LPC	Laboratory pasteurization count
MA	MacConkey agar
PIC	Preliminary incubation count
PPM	Part per million
SCC	Somatic cell count
SMEDP	Standard methods for examination of dairy products
SPC	Standard plate count
TAC	Total aerobic count
UHT	Ultra high temperature

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Background

Bacteria in raw milk are a concern for processors and consumers of milk and milk products. The composition and the number of bacteria in raw milk affect the quality and safety of dairy products. High microbial counts in raw milk are responsible for quality defects in pasteurized milk, ultra high temperature (UHT) pasteurized milk, dried skimmed milk, butter and cheese (1-3). Additionally several human microbial pathogens have been found in milk and milk products including, *Listeria monocytogenes, Salmonella spp., Staphylococcus aureus, Campylobacter jejuni, Brucella* spp., and *Mycobacterium tuberculosis* (4, 5). Therefore, many jurisdictions include limits on the total number of bacteria in raw milk to ensure quality and safety of the final product.

Studies have shown that examining bulk tank milk (BTM) is useful for diagnosing multiple problems in dairy herds in relation to milk quality. Therefore, raw BTM is often subjected to a number of bacteriological tests that are used as indicators of udder health, milk harvest hygiene and storage conditions on the farm (6-8). These tests include, standard plate count (SPC) or its alternative total aerobic count (TAC), preliminary incubation count (PIC), laboratory pasteurization count (LPC) and coliform count (CC). Among these tests, SPC is the most frequently used in regulatory programs and reflects the general hygienic condition during milk production, while each of the other tests identify potential contamination sources of concern for milk quality (6-9).

This chapter will review the microflora of raw milk, various sources of contamination of raw milk, and bacteriological tests that are used for assessment of bacterial contamination of raw milk.

1.2 Raw milk microflora

Milk is an excellent substrate for growth of many microorganisms, including pathogenic and spoilage bacteria. The level and composition of the microflora of raw milk provide information on the hygiene level during milk production. The main groups of raw milk bacteria include: psychrotrophs, thermodurics, coliforms, and mastitis pathogens

1.2.1 Psychrotrophic bacteria

Psychrotrophs are those microorganisms able to grow at 7 °C and below, irrespective of their optimum growth temperature (10). The most commonly occurring psychrotrophs in raw milk are Gram-negative rods of which *Pseudomonas* spp., accounts for at least 50% (11). Other genera which are more rarely encountered include *Alcaligenes, Aeromonas, and Acinetobacter* (10). Psychrotrophs are generally found in water, soil and vegetation. They are introduced into the milk as a result of contamination of milking equipment by these sources. If milk is produced under proper sanitary conditions, psychrotrophs will comprise less than 10% of the microflora of freshly drawn milk. Under unsanitary production practices, psychrotrophs may constitute more than 70% of the total bacterial count (12).

Psychrotrophs generally do not survive pasteurization, therefore their presence in processed milk indicates either improper pasteurization or post-pasteurization contamination (1, 10). Elevation in raw milk psychrotroph counts is of concern for two reasons. Although most psychrotrophs do not survive pasteurization, they may produce extracellular heat resistant lipases and proteinases. The lipolytic and proteolytic activities of these enzymes reduce the shelf life and quality of milk and milk products.

Additionally, some bacterial species, including spore-formers (*Bacillus* spp., and *Clostridium* spp.) and non-spore formers (*Microbacterium, Micrococcus,* and *Corynebacterium*) have both psychrotrophic and thermoduric characteristics. Psychrotrophic spore-formers are of particular interest as spoilage-causing agents because they can survive pasteurization, germinate, and multiply at storage temperature of pasteurized products (1, 13-15).

1.2.2 Thermoduric bacteria

Thermodurics are microorganisms (vegetative cells or spores) that survive pasteurization conditions. Thermoduric bacteria isolated from milk include *Micrococcus, Microbacterium, Streptococcus, Lactobacillus, Corynebacterium, Bacillus,* and *Clostridium* (10). *Microbacterium lacticum* and spore-formers (*Bacillus* spp., and *Clostridium* spp.) show strong resistance to high temperature and survive pasteurization at 63 °C for 30 min. Species of *Streptococcus, Lactobacillus*, and *Corynebacterium* are less heat resistant, with <1% of a given population surviving heat treatment of 63 °C for 30 min (16).

Meer et al. (1) reported that 25 % of all of the shelf-life problems of pasteurized milk and cream products in the USA may be caused by thermoduric psychrotrophs. Chen et al. (17) reported that 86 % of thermoduric psychrotrophic bacteria isolated from raw milk were *Bacillus* spp. The sources of *Bacillus* spores in the dairy farm environment include silage (18), pasture (19), soil (20), and bedding material (11, 21).

1.2.3 Coliform bacteria

Coliform bacteria are aerobic and facultative anaerobic, Gram-negative rods, which ferment lactose with the production of acid and gas. Genera classified as coliforms include *Escherichia, Klebsiella, Citrobacter*, and *Enterobacter* (10).

Coliforms are ubiquitous in the farm environment and can be found in fecal and bedding material. Another potential source of coliforms and other Gram-negative bacteria in BTM is the water used for cleaning the milking equipment (16). High levels of coliforms in raw milk usually reflect unhygienic production practices (6-9). Coliforms are easily killed by pasteurization, therefore their presence in processed milk indicates either inadequate pasteurization or post-pasteurization contamination.

1.2.4 Mastitis pathogens

Mastitis pathogens in BTM can be contagious or environmental. The contagious mastitis pathogens *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma* spp. reside primarily in the cow's udder, therefore, when they are found in bulk milk, they indicate the presence of intramammary infections in the herd (22). Environmental bacteria, such as *Streptococcus uberis, Streptococcus dysgalactiae*, and coliforms, may enter milk from intramammary infections, but also from nonspecific contamination such as skin surface, manure, and bedding material (16). The presence of these organisms in BTM may relate to the general level of environmental and milking hygiene (23).

1.3 Sources of microbial contamination of raw milk

There are three main sources of microbial milk contaminants on the farm: from within the udder, from the exterior of udder, and from the surfaces of milking equipment (16). Another factor of importance in determining the bacterial load of raw milk is the

efficiency of milk cooling procedures. Although cooling does not contribute bacteria directly to milk, it does influence the total bacterial count by modifying the rate of bacterial growth during storage of milk.

1.3.1 Contamination from within the udder

Milk drawn from a healthy cow has a low bacterial count, generally less than 1,000 cfu (colony forming unit)/ml, which originate from the teat canal. Therefore, microflora of a healthy udder do not cause significant increases in bulk tank total bacterial count (6). The influence of mastitis on bacterial count of BTM depends on the size of the herd, number of infected cows, and the ratio of mastitic to nonmastitic milk (24). Although cows with sub-clinical mastitis have been reported to contribute $< 10^4$ cfu/ml to total bacterial count, cows with clinical mastitis may elevate bulk tank count up to 10^5 cfu/ml (11).

Cows infected with *Streptococcus uberis* can shed up to 10^7 cfu/ml into their milk (25), and cows infected with *E. coli* can shed up to 10^8 cfu/ml (26). Therefore, one infected cow can influence total bacterial numbers of whole BTM especially in small sized herds.

The presence of *Staphylococcus aureus* mastitis in a herd will not likely elevate the total bacterial count because *S. aureus* is shed in relatively low numbers into milk (6). A Scottish study by Jeffrey and Wilson (27) of 754 BTM samples with total bacterial count > 45,000 cfu/ml, showed that 43.8 % of the samples had bacterial loads that were dominated by mastitis microorganisms. In a Danish study, mastitis pathogens were found in 48% of milk samples with total bacterial count >30,000 cfu/ml and were the main cause of elevated microbial count in 8% of the samples (28). Hayes et al. (24) reported

clinical and subclinical infection with *S. uberis* to be the cause of elevated total bacterial count in BTM in samples in which other environmental contaminants such as *E. coli, Klebsiella* spp., and *Bacillus* spp. were absent. A recent study in New York State showed that streptococci were important contributor to the total bacterial count in BTM and may be present at levels >100,000 cfu/ml. (29).

1.3.2 Contamination from the exterior of the udder and teats

Between milkings, teats and udder often become soiled with manure and bedding materials. If the teats were not thoroughly cleaned and dried before milking, this dirt with the associated microorganisms will be transferred into the milk (11). Contamination from the exterior of the udder and teats can contribute environmental microorganisms such as streptococci, staphylococci, spore-forming bacilli, coliforms, and other Gram-negative bacteria. The bacterial count of the exterior of the udder and teats is influenced by bedding management, whether cows have access to pasture or not, and cow preparation before milking.

Previous studies indicated that bacterial counts in inorganic bedding (sand and limestone) are usually lower than those in organic (sawdust and straw) bedding (30). Bacterial populations on the teat surface have been found to be correlated with those in bedding material, and high bacterial counts in bedding expose the teats to high bacterial numbers and increase the risk of mastitis due to environmental pathogens (30, 31).

Several methods have been developed for scoring the cleanliness of dairy cows and the degree of contamination of different body areas, including the udder and teats, with dirt and bedding material (32-34). These studies reported associations between poor udder hygiene and milk quality and udder health as measured by SCC. For example,

using a four-point udder hygiene scoring scale clean score equaled 1or 2 and dirty score equaled 3 or 4, Schreiner and Ruegg (32) have shown that somatic cell counts and the prevalence of intramammary environmental pathogens were higher for dirty animals. The study revealed that dirty animals were 1.5 times more likely to be infected with a major mastitis pathogen than clean animals.

For herds using pasture, dirty udders are more likely to occur in winter, when the cows are housed, than in summer, when the cows are on pasture. In a study evaluating teat end microflora, populations of environmental pathogens on teat ends were lower on pastured cattle than on confined cattle (35). Bulk tank milk bacteria counts also have been shown to be lower when cows are pastured than when confined (36, 37).

Teat end sanitation is important in reducing the number of bacteria at the teat end before attaching the milking unit, and thus aids in the control of mastitis and improves milk quality (38). Several studies have shown that premilking teat disinfection can reduce bacterial counts on the teat surface (39, 40) and in milk (8, 41, 42). On the other hand, some studies reported no association between the teat cleaning regime and bacterial counts in milk (43, 44).

1.3.3 Contamination from the surfaces of the milking equipment

Proper cleaning and sanitation of the milking system is one of the most important aspects of producing high quality raw milk. Cleaning of the milking system involves a combination of chemical, thermal, and physical processes. A balance between cleaning temperature, time, chemical concentration and mechanical action is essential for successful removal of milk residues. A deficiency in any of these parameters could result in build-up of milk residues which provides nutrients for growth and multiplication of

bacteria between milking times (11, 45). The build-up of milk residues and the associated bacterial growth commonly occurs at poorly designed and difficult-to-clean parts of the milking system such as crevices, joints, and blind ends (6, 45).

Other factors that could interfere with cleaning efficiency and the associated bacterial growth include failure to replace worn rubber components and the development of cracks and rough surfaces on liner surfaces and rubber gaskets (45, 46). In general, environmental contaminants are more likely to grow on contaminated equipment surfaces than are organisms associated with mastitis infections (16).

The temperature of cleaning and sanitizing solutions affects the type of microorganisms on milking equipment surfaces. A predominance of thermoduric bacteria over other microflora from the milking equipment could be due to using high temperature during equipment cleaning, without adequate chemical cleaning. On the other hand, insufficiently hot water (<42 °C) when associated with improper sanitization allows the dominance of thermolabile species such as *Pseudomonas* spp., and coliform bacteria (6, 16, 43).

The quality and quantity of the water used for cleaning the milking machine has a significant impact on cleaning efficiency. According to Bramley and McKinnon (16) untreated water could be a source of contamination of milking equipment with *Pseudomonas* spp., coliforms and other Gram negative bacteria. Additionally, hard water can reduce the effectiveness of cleaning chemicals, and may also lead to the formation of films or deposits on the milking system (47).

The efficiency of the cleaning process can be assessed by visual inspection of residual films on milk contact surfaces, bulk milk culture, and ATP bioluminescence

(45). ATP bioluminescence has been developed to assess the effectiveness of cleaning and sanitation of food contact surfaces including the milking system. This technique involves surface swabbing, followed by reaction of any ATP with luciferin and luciferase enzyme, resulting in the emission of light which is detected and quantified as relative light units (RLU) by a hand-held luminometer. The ATP method detects both microbial and non-microbial (organic debris and milk residues) sources of ATP (48). The ATP method is fast (less than 5 minutes) and simple as opposed to the culture method (2-3 days incubation). There is considerable variation in the ATP data, and hence this method must be used carefully and with sufficient number of ATP swabs to obtain meaningful results (48).

1.3.4 Effect of storage time and temperature on bacterial count

Proper cooling of raw milk after its collection from the udder is a major factor in controlling bacterial growth during storage. Milk should be cooled to 4.4 °C or less within 30 min of milking, and during subsequent milking, the blend temperature should be kept below 7.2 °C (49).

Although refrigerated storage of raw milk is required for extending the shelf-life and eliminating spoilage by mesophilic bacteria, it supports the growth of psychrotrophic bacteria (50). If the number of psychrotrophs exceeded 3-5 x 10^6 cfu/ml, they could result in spoilage of milk due to production of lipolytic and proteolytic enzymes (50, 51). *Pseudomonas* spp usually account for 50 % of psychrotrophs and have the shortest generation time at refrigeration temperature (2, 52). The generation time of *Pseudomonas fluorescens* had been reported to be 30.2 h at 0 to 2 °C, 6.7 to 7.2 h at 4°C to 6 °C, and 1.4 h at 20 °C (53). In addition to the ability to grow at low temperature, *Pseudomonas*

fluorescens can also secrete adhesive exopolysaccharides which could facilitate biofilm formation and subsequent protection from the effect of sanitizers (54).

Delayed or inadequate cooling will increase bacterial counts of milk, and this increase depends on the temperature and length of storage, and the initial number and type of microorganisms (11). Bacterial count increases with increased storage temperature. The growth rate of most bacteria is inhibited below 2 °C, however their numbers increase rapidly at a temperature above 10 °C (51). Griffiths et al. (55) reported that decreasing the storage temperature from 6 to 2 °C had increased the time for psychrotrophic count to reach 10^6 cfu/ml from 2.9 to 5 days. Similarly, Haryani et al. (56) reported that the average time taken for psychrotrophic count to reach 10^7 cfu/ml at 2 °C , 4 °C, and 7 °C were 9 , 7, and 4 days, respectively.

Bacterial growth during storage is also influenced by the initial microbial quality of raw milk. Guinot-Thomas et al. (57) reported that a milk of good quality (4×10^3 cfu/ml) can be stored for 48 h at 4 °C without significant increase in psychrotrophic count. In contrast, low quality milk (2.8×10^4 cfu/ml) showed a significantly higher psychrotrophic count after 48 h storage at 4 °C (5.1×10^6 cfu/ml).

1.4 Tests for evaluation of bacterial contamination of raw milk

1.4.1 Standard plate count

The SPC provides an estimate of the total number of aerobic bacteria present in raw milk. The SPC is determined by plating a diluted milk sample onto standard method agar followed by aerobic incubation for 48 h at 32 °C, after which bacterial colonies are counted and the number expressed as cfu/ml. Other methods used as alternatives to SPC

include: petrifilm aerobic count, plate loop count, and spiral plate count (10). The petrifilm aerobic count method is a ready-made plating system which measures all bacteria able to form colonies on a nutrient medium embedded in a plastic film within 48 h at 32 °C. This method can be used for raw and pasteurized milk and produces results that are not significantly different from the SPC method (10). The spiral plate count method use a spiroplater to deposit a small volume of milk (50 μL) on the surface of a rotating agar plate with more sample in the center and less towards the plate edge. This method also produces comparable results to the SPC method (10). Bactoscan is a more recent method that utilizes fluorescent staining to count individual bacterial cells/ml rather than colony forming units (cfu/ml), which results in higher counts than other counting methods (4). In our study, the petrifilm culture system was used for estimation of the TAC, PIC, LPC, and CC levels in milk.

The SPC does not specify the source of bacterial contamination or identity of microbial groups leading to high counts, its main value is to indicate changes in the production, collection, handling, and storage environment (11).

The SPC of raw milk can range from less than 10^3 cfu/ml to more than 10^6 cfu/ml. In the United States, the legal maximum based on the Pasteurized Milk Ordinance is $<10^5$ cfu/ml, although most industry standards require a count $<5x10^4$ cfu/ml (49). In Canada, the legal maximum is $5x10^4$ cfu/ml (58). A count less than $5x10^3$ cfu/ml indicates proper sanitation and cooling, whereas a value of more than 10^5 cfu/ml is evidence of serious defects in production hygiene. Most producers can achieve a count of less than 10^4 cfu/ml if all aspects of hygiene are closely watched (7, 59).

As the SPC increases, the distribution of microflora shifts from being dominated by micrococci and streptococci to being dominated by Gram-negative rods (11).

Research data on the specific on-farms risks associated with elevated SPC is sparse, however multiple sources, including improper cleaning and sanitizing of dairy equipment, milking dirty, wet cows, inadequate cooling, and clinical and subclinical mastitis problems have been implicated as potential bacterial origins (6,7, 59).

1.4.2 Preliminary incubation count

This procedure estimates the number of psychrotrophic or cold-loving bacteria. The test is done by holding milk at 12.8 °C for 18 h, followed by SPC procedures (60). The value of this test lies in its comparison with the SPC to determine if any significant increase in bacterial numbers has occurred during the holding period. The PIC count should be less than $5x10^4$ cfu/ml and not more than 3-4 times the SPC (46). A desirable level for PIC is less than 25,000 cfu/ml (49).

A high PIC could be associated with inadequate cleaning and sanitation of the milking equipment, poor udder preparation, contaminated water supply, and improper cooling or prolonged storage of milk (6, 7, 49).

1.4.3 Laboratory pasteurization count

This test quantifies the number of thermoduric bacteria by subjecting raw milk to a pasteurization procedure, typically 62.8 °C for 30 min, and quantifying the surviving organisms. This process kills most bacteria present in milk, including mastitis-causing bacteria, however, certain species may survive in low numbers. These surviving bacteria may cause off flavors and reduce shelf-life of dairy products (7, 46, 59).

A laboratory pasteurization count over 200 cfu/ml is considered high, a count between 100-200 cfu/ml indicates adequate cleaning and sanitation of the milking system, and a count less than 10 cfu/ml indicates excellent equipment hygiene (7, 46, 59).

1.4.4 Coliform count

The coliform count estimates the number of coliform bacteria in milk. Coliform count may be determined by plating a milk sample onto Violet Red Bile Agar followed by incubation at 32 °C for 24 h after which colonies are counted as cfu/ml (10).

Coliform counts serve as an indicator of both the effectiveness of udder preparation procedures prior to milking and the cleanliness of the cow's environment (9). While most risk of high CC is attributed to cow and environmental factors, coliform bacteria can incubate in milk residues left on milking equipment cleaned at low temperature and thereby become a major source of contamination of BTM (9, 36). A sporadic high coliform count could also be associated with unrecognized coliform mastitis (11).

A coliform count should be less than 50 cfu/ml, and counts between 100-1000 cfu/ml are indicators of poor milking hygiene. A count of more than 1000 cfu/ml suggests incubation of bacteria in milk residues on milking equipment. A count less than 10 cfu/ml indicates excellence in both pre-milking hygiene and equipment sanitation (6, 59).

1.5 Objectives of thesis and thesis outline

Although it is well recognized that good quality raw milk is essential for producing quality milk and milk products, there is limited information available on the

influence of management factors on bulk tank bacterial counts. Additionally, no observational studies have been conducted to evaluate multiple risk factors, and the interactions that might exist between these factors that could affect the quality of raw milk. Therefore, the overall objective of this research was to investigate the association between on-farm management practices and bacteriological quality of bulk tank milk as measured by total aerobic, preliminary incubation, laboratory pasteurization and coliform counts.

The specific objectives addressed in this thesis include the following:

1. Conduct a descriptive study to determine the current level (mean, median and percentiles) for each of the milk quality parameters in PEI and compare these levels with other regions using similar testing. This objective has been addressed in Chapter 2 "Microbiological Quality of Bulk Tank Raw Milk in Prince Edward Island Dairy Herds"
2. Relate laboratory test results to on-farm management practices. To achieve this goal, a mail-out survey was sent to all dairy producers in PEI to collect information on different aspects of hygiene and management practices at the farm. The survey data are presented in Chapter 3 "The Association Between Bulk Tank Milk Analysis for Raw Milk Quality and On-farm Management Practices". Additionally, a case control study was implemented in which cases and controls were defined based on laboratory test results and the exposure status of both case and control farms was evaluated by highly trained technicians. On-farm evaluation included observation of basic management practices, extensive analytical evaluation of equipment hygiene and cooling efficiency, and scoring of

cow and environmental hygiene. The results from the case-control study are presented in Chapter 4 "Risk Factors for Bacteriological Quality of Bulk Tank Milk in Prince Edward Island Dairy Herds. Part 1: Overall risk factors" and in Chapter 5 "Risk Factors for Bacteriological Quality of Bulk Tank Milk in Prince Edward Island Dairy Herds. Part 2: Bacterial count-specific risk factors".

3. Identify bacteria which survive laboratory pasteurization and characterize their lipolytic and/or proteolytic activity. These data are presented in Chapter 6 "Identification and Characterization of Lipolytic and Proteolytic Bacteria in Pasteurized Milk".
4. Speciation of BTM cultures to identify mastitis pathogens which could be associated with elevated bacterial count in BTM. The results of this study are presented in Chapter 7 "Mastitis Pathogens Associated With Elevated Bacterial Count in Bulk Tank Milk of Prince Edward Island Dairy Herds".

Finally, the overall discussion and conclusions are presented in Chapter 8.

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CHAPTER 2. MICROBIOLOGICAL QUALITY OF BULK TANK RAW MILK IN PRINCE EDWARD ISLAND DAIRY HERDS

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2.1 Abstract

The objectives of this study were to evaluate microbiological quality of bulk tank milk in Prince Edward Island, to evaluate correlation among milk quality criteria, and to determine seasonal effects on milk quality parameters. Bulk tank raw milk quality was evaluated on all Prince Edward Island dairy herds (n = 235) over a two year period (March 2005 to March 2007). Biweekly total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC) and coliform (CC) counts were determined using a Petrifilm culture system. Additionally, bulk tank somatic cell count (BTSCC) was done on a weekly basis.

The mean and median values were 12.8×10^3 and 4.9×10^3 cfu/ml for TAC, 29.6×10^3 and 13×10^3 cfu/ml for PIC, 87 and 12 cfu/ml for LPC, 21 and 5 cfu/ml for CC, and 218×10^3 and 187×10^3 cells/ml for SCC, respectively. There was moderate correlation (0.57) between TAC and PIC. All other correlation coefficients were low (<0.26). Correlation results suggested that a single quality parameter could not predict others used in this study. Seasonal data indicate that: 1) in general, all counts tended to be low in winter 2) the CC and SCC were always higher in summer, and 3) the TAC tended to be higher during summer.

2.2 Introduction

Bacteria in raw milk can affect the quality, safety and consumer acceptance of dairy products. Several human microbial pathogens, such as *Listeria monocytogenes, Salmonella* spp., *Staphylococcus aureus, Campylobacter jejuni*, and *Mycobacterium tuberculosis,* have been found to be associated with milk and milk products (1, 2). Even for organisms that are not pathogenic to humans, there can be effects on milk quality. For example, high microbial counts in raw milk are responsible for quality defects in pasteurized milk, UHT milk, dried skimmed milk, butter and cheese (3, 4). Additionally, selecting raw milk of high quality has been associated with a drop in consumer complaints due to fluid milk quality (5). As a result, many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure quality and safety of the final product.

Microbiological quality of bulk tank milk (BTM) is measured by means of several tests including, total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC) and coliform (CC) counts. The TAC is an alternative to the standard plate count (SPC) and provides an estimate of the total number of aerobic bacteria present in raw milk. This measure does not provide information about specific hygienic failure or the identity of specific microbial groups in the milk, it indicates changes in the production, collection, handling, and storage environment (6). The PIC measures the number of psychrotrophic or cold-loving bacteria which grow at poor refrigeration temperature. The PIC is generally higher than the TAC, and a count 3 times greater than the TAC is considered significantly elevated (7). The LPC quantifies the number of thermoduric bacteria which survive laboratory pasteurization at 62.8 °C for 30 min. This

process kills most bacteria present in milk, including mastitis-causing bacteria and zoonotic pathogens, however, certain species may survive in low numbers. These surviving bacteria may cause off flavors and reduced shelf-life of dairy products. The number of coliforms (CC) in milk is an indicator of the cleanliness of cows and their environment (7, 8). Bulk tank somatic cell count (BTSCC) is an indicator of the level of subclinical mastitis present in a herd. Milk with high BTSCC has a higher level of proteolytic and lipolytic enzymes, which reduce cheese production and affect the flavor and shelf-life of dairy products (4).

With the exception of SPC (or its alternative TAC) and BTSCC, these tests were not routinely performed in Prince Edward Island (PEI) prior to the initiation of the project. As part of a program to improve raw milk quality, the industry wanted to establish benchmark values on a province-wide basis to determine current levels for each test. Additionally, literature regarding seasonal effects on microbial quality of BTM is scarce, so the objectives of this study were: 1) determination of the current bacteriological quality of BTM in PEI dairy herds using several quality parameters 2) evaluation of correlations among quality parameters, and 3) investigation of seasonal variations in BTM quality parameters.

2.3 Materials and Methods

2.3.1 Data collection

Bulk tank milk samples were collected from all PEI dairy herds (n = 235) over a two year period (March 2005-March 2007). Raw milk samples were collected from fifty percent of herds on alternating weeks, so that each herd was sampled approximately

every two weeks. Samples were collected in 30 mL sterile screw cap tubes (Starplex Scientific Inc., Etobicoke, Ont.) by trained milk haulers and held on ice until arrival at the laboratory. All microbiological analyses were performed within 36 h of pick-up at the farm.

2.3.2 Bacteriological analysis of BTM

Bulk tank milk samples were examined for TAC, PIC, LPC, and CC using Petrifilm (3M Canada, London Ontario) at the Atlantic Veterinary College. Milk samples were mixed thoroughly by vortex. For TAC and PIC, the plate loop (PLC) counting method was used with 1/100 dilution. The alternative (PLC) method was validated according to Standard Methods for the Examination of Dairy Products (SMEDP) procedures (9). For LPC and CC, one ml of milk was cultured directly on Petrifilm. The TAC, LPC and CC counts were conducted according to SMEDP (2004). The PIC was performed as described by Richardson (10). Plates for enumeration of TAC, PIC, and LPC were incubated at 32 °C for 48 h. Plates for CC were incubated at 32 °C for 24 h. All plates were read using an automated counter (3M Petrifilm Plate Reader, 3M Canada, London Ontario) and data were stored in Excel spreadsheets (Microsoft, Seattle, WA) before merging into the database (below). The maximum reading by the automated reader is "> 999", which corresponds to a minimal bacterial load of 100,000 in the case of TAC and PIC, and 1,000 in the case of LPC and CC. All plate counts were expressed as the number of cfu/ml.

In addition to bacterial count, SCC was evaluated on a weekly basis using a CombiFOSS 4000 or CombiFOSS 6000 FC (FOSS Electric, Hillrød Denmark) at the PEI milk quality laboratory.

2.3.3 Statistical analysis

Data were collected on spreadsheets and merged into a single database using Stata version10 (Stata Corp, College Station TX). Summary statistics, frequency distributions and Spearman's rank correlations were computed on raw data. The consistency of the results within herds was evaluated by calculation of the coefficient of variation (CV) for each test within herds after natural logarithmic transformation of all counts. The CV is the ratio of the standard deviation to the mean. For seasonal variation, the bacterial counts were categorized into 4 seasons by study year: spring (March 21st to June 20th), summer (June 21st to September 20th), fall (September 21st to December 20th) and winter (December 21st to March 20th). Study year one started on March 21st 2005 and completed March 20th 2006, while study year two extended from March 21st 2006 to March 27th 2007. A graph showing the median, 25^{th} and 75^{th} percentiles was presented for each quality parameter by season and study year. Statistical significance of seasonal effects were evaluated using linear mixed models (11) for mean values on a logarithmic scale, but results are presented as medians and inter-quartile ranges on the original scale.

2.4 Results

2.4.1 Descriptive Statistics

A total of 11,099 BTM samples were evaluated for each of TAC, PIC, LPC, and CC counts, and 22,714 samples for SCC. Table 2.1 and figure 2.1 show summary statistics and frequency distributions for each of the bacterial parameters and SCC. For all 5 parameters, the mean count was larger than the median, indicating a right skewed distribution. Each count was categorized into intervals based on industry-based quality

thresholds to illustrate the distribution and to allow comparison of the results with other studies which used similar testing and intervals. Thresholds for high bacteria counts were selected based on suggested guidelines by Murphy (7). The bonus threshold was determined by local industry, based on average bacterial and SCC counts over a 3 month period. A comparative summary of the results of this study and other studies using similar testing is presented in table 2.2.

Fifty percent of BTM samples had TAC ≤4,900 cfu/mL, approximately 80% were below the bonus level (15,000 cfu/ml) and 6.2% were above the regulatory limit for total bacterial count in the province (50,000 cfu/ml). The median PIC was 13,000 cfu/ml, 64% of the samples were below the bonus level (25,000 cfu/ml) and 23% were considered high (>50,000 cfu/ml).

Fifty percent of the samples had LPC ≤ 12 cfu/mL, 90% had count below the bonus level (100 cfu/ml), and 8% exceeded the threshold for high count (200 cfu/ml). The median CC was 5 cfu/ml, 71% of the samples were below the bonus threshold (25 cfu/mL) and 11% were considered high (>50 cfu/ml). Fifty percent of BTM samples had SCC ≤ 187,000 cells/ml, 55% were below the bonus level (200,000 cells/ml), and 3.9% were above the regulatory limit for SCC in the province (500,000 cells/ml).

The percentage of herds which had no test over the threshold varied widely among milk quality tests and ranged from 1.27% for PIC to 28.03 % for SCC (Table 2.3). For PIC, about 28% of the herds had 30% of their PIC tests over the threshold.

2.4.2 Correlation between Milk Quality Parameters

The correlation coefficients between milk quality parameters are shown in table 2.4. All parameters had positive correlations, and the highest correlation was between

TAC and PIC (0.57), while the poorest correlation was between PIC and SCC (0.08). All other correlation coefficients were low (<0.26).

2.4.3 Coefficient of Variation for Milk Quality Parameters

The result of the CV for each of the quality parameters is shown in table 2.5. The lowest CV was for SCC (0.03), whereas, the highest CV was for CC (0.50). Additionally, for all quality parameters, the CV decreased with increases in the mean count (Figure 2.2).

2.4.4 Seasonal Variation

Overall, there was a significant effect of season on each of the quality parameters ($P < 0.001$). In addition, there was a difference ($P < 0.001$) between year1 and year 2 for TAC, PIC, and SCC. The interaction between season and year was significant ($P < 0.05$) for all quality parameters except SCC ($P = 0.197$) (data not shown).

Figure 2.3 shows the median and interquartile range for different bacterial counts and somatic cell count by season and year. In year 1, the median TAC was highest during summer (6,850 cfu/ml), whereas, in year 2, the median observed during fall was equivalent to that observed in the summer, 7,700 and 7,600 cfu/ml, respectively. In both year 1 and year 2, the lowest median count was observed during winter, 3,600 and 3,200 cfu/ml, respectively. For PIC, the median and 75th percentile were highest during spring, 17,000 and 60,500 cfu/ml, respectively in year 1. In year 2, the median and 75th percentile during spring were similar to those observed in year 1, however, the highest median and 75th percentile were observed during fall, 19,000 and 68,000 cfu/ml, respectively.

For LPC, in year 1, the median and 75th percentile were highest during summer, 21 and 51 cfu/ml, respectively. In year 2, the median LPC was approximately the same among seasons, ranging from 8 to 12 cfu/ml, however, the 75th percentile was higher during fall and winter at 30 cfu/ml. The median and 75th percentiles for CC were highest during summer in both years. The highest median and 75th percentile were observed in summer year 1, 10 and 31 cfu/ml, respectively, whereas the lowest median and 75th percentile were in winter year 2, 3 and 8 cfu/ml, respectively.

The BTSCC showed a similar pattern to coliforms with higher median and percentiles during summer in both years compared to other seasons. The highest median and 75th percentile were observed in summer year 2, 208,000 and 305,000 cells/ml, respectively, whereas the lowest median and 75th percentile were in spring year 1, 170,000 and 257,000 cells/ml, respectively.

2.5 Discussion

High quality dairy products start with high quality raw milk. Because poor quality raw milk leaving the farm cannot be transformed into a high-quality product for the consumer, there are continuing demands upon producers to improve their raw milk bacteria and BTSCC numbers. Many processors provide incentive programs to encourage dairy farmers to produce milk with lower bacteria and BTSCC than the required regulatory limits. The TAC and SCC are the only regulatory tests among the 5 parameters used in this study, however, they do not give a full assessment of the hygienic quality of raw milk. Therefore many jurisdictions use several tests to assess raw BTM quality. In this study, 5 criteria were used, TAC, PIC, LPC, CC, and SCC.

2.5.1 Descriptive Statistics

Total Aerobic Count: The TAC (alternative to SPC) is an indicator of the general hygienic condition during milk production, collection, and storage. A count of less than 5,000 cfu/ml indicates proper hygiene, and a count of <10,000 cfu/ml should be achievable by most farms (7, 8). In our study approximately 50% of the samples were below 5000 cfu/ml and 71% were <10,000 cfu/ml. The percentage of herds achieving low bacteria levels was much higher than those reported by Boor et al. (12) in New York State and somewhat lower than those reported by Jones and Summer (13) in Virginia and by Jayarao et al. (14) in Pennsylvania (Table 2.2). Additionally, previous studies in the United States, reported variation in geometric mean SPC that ranged from 4,700 to 17,000 cfu/ml (12, 15). Our geometric mean (5,300 cfu/ml) was close to the lower end of the range reported in these studies. However, the right tail of our data was truncated at 100,000 cfu/ml for both TAC and PIC and 1,000 cfu/ml for LPC, which will affect mean counts (lower) but not percentiles. These variations in SPC among different regions indicate that it can be influenced by different management practices.

Preliminary Incubation Count: The PIC is used to estimate the number of psychrotrophic bacteria in raw milk. An acceptable PIC count should be <50,000 cfu/ml and not more than 3 to 4 times the SPC (7) and a desirable PIC count is <25,000 cfu/ml (13). In our study, approximately 64 and 77% of the samples had PIC <25,000 and 50,000 cfu/ml, respectively. The percentage of herds achieving good or acceptable PIC levels was 35 and 32 percentage points higher, respectively than those reported by Boor et al (12). In this study 16% of the samples with PIC >50,000 had PIC three or more times higher than TAC, indicating a high psychrotroph burden. Our geometric mean for

PIC was also much lower than that reported in New York and was in the lower range reported by Peeler et al. (15) in the multi-state study. Generally, high levels of psychrotrophic bacteria in raw milk will contribute significant quantities of heat stable proteases and lipases that will break down protein and fat after pasteurization (3) and cause spoilage of the final product during storage.

Laboratory Pasteurization Count: The LPC identifies thermoduric bacteria that can survive exposure to pasteurization temperatures. A count between 100-200 cfu/ml indicates adequate cleaning and sanitation of the milking system, and a count of less than 10 cfu/ml indicates excellent equipment hygiene (16). The great majority of our samples (90%) were < 100 cfu/ml, whereas, in New York State, only 44% of BTM samples were < 100 cfu/ml. Additionally, the geometric mean and the median LPC reported in this study were also lower than those reported in New York State and in Pennsylvania. High thermoduric counts in bulk tank milk are mainly associated with the presence of heat tolerant bacteria on milking equipment (7, 16).

Coliform Count: Coliforms are used as indicators of unsanitary production practices. A count of less than 50 cfu/ml is considered acceptable. In our study, 89% of BTM samples had CC <50 cfu/ml, whereas in New York and Pennsylvania milk samples, 39% and 45% were <50 cfu/ml, respectively. Additionally, our median and geometric mean CC were lower than those reported in New York and Pennsylvania.

Bulk Tank Somatic Cell Count: The mean BTSCC was 218,000 cells/ml (geometric mean of 184,000 cells/ml) and 50% of the samples were <187,000 cells/ml. In Ontario, Sargent et al (17) reported a mean BTSCC of 250,000 cells/ml. According to Canadian Dairy Commission, the mean BTSCC in Canadian provinces in 2006 ranged

from 155,000 cells/ml (British Columbia) to 268,000 cells/ml (Saskatchewan) with PEI ranked third (221,000 cells/ml) after British Columbia and Alberta (18). In the United States, the average BTSCC was 363,000 cells/ml in New York (19), whereas in Pennsylvania, the mean BTSCC was 315,000 cells/ml and the median was 348,000 cells/ml (14).

Recent published BTSCC data from outside North America indicate that the geometric mean BTSCC in Ireland in 2004 was approximately 251,000 cells/ml (20) , whereas in Norway the geometric mean was 115,000 cells/ ml (21).

Ma et al. (22) reported that high SCC raw milk had more lipolysis and proteolysis than low SCC raw milk. They also indicated that higher enzymatic activity in high SCC raw milk affected the quality of pasteurized fluid milk by accelerating the development of sensory defects such as rancidity and bitterness.

In general, bacterial counts reported in this study were lower than those reported in New York and Pennsylvania (12, 14) and were in the lower end of the range reported by Peeler et al. (15) in the study which involved 11 states. Additionally, the mean BTSCC reported here was lower than the means reported in the United States (14, 19) and Ireland (20) and higher than the mean reported in Norway (21). These variations may be attributed to the use of different management practices (23).

2.5.2 Correlation between Quality Parameters

The TAC was moderately correlated with the PIC (0.57). The correlation between other quality parameters was low <0.26. Boor et al. (12) reported low to moderate correlations between various quality parameters, for example, the correlation coefficient between SPC and PIC was 0.58 in their study. In addition, the correlation between SCC

and different bacterial count was low. This was also similar to that reported by Jayarao et al. (14). The weak correlations reported in this study and previous studies suggest that each count gives different information in relation to management practices and sources of bacterial contamination. It also substantiates the conclusion by other authors that one bacterial test could not be used to estimate other tests (12, 24).

2.5.3 Coefficient of Variation for Milk Quality Parameters

The CV varied widely among milk quality parameters. The values of the CV indicate reasonable consistency of the results for SCC, moderate variation in TAC and PIC and high variability in LPC and CC. Some of the variation in test results could be attributed to seasonal effects and changes in management practices during the study. However, overall, the relatively high CVs indicate that herd assessments should not rely on a single measurement of any of these parameters in time.

2.5.4 Seasonal Variations

The results regarding seasonal variation showed that there was no consistent seasonal pattern over the 2 year study period for TAC, PIC, and LPC, however, these quality parameters tended to have low median counts during winter. On the other hand, coliform and SCC counts showed similar pattern in both years, with the median counts being highest during the summer. High somatic cell and total bacterial counts during summer months were also reported in the United States (19, 25). However, in our study, the TAC showed a second peak in the fall of year 2. To investigate the reason for the fall peak, we looked at the data on a monthly basis. This analysis (not shown) revealed peaks in median TAC and PIC during September and November of year 2 compared to the

previous year. We examined meteorological data for the 2 years and found no substantive variation in ambient temperature and precipitation between the 2 years.

In Ontario, Schukken et al. (26) and Sargent et al. (17) reported a significant seasonal pattern for SCC, where the lowest mean occurred in April, and the highest mean occurred in October, however, there was no clear seasonal effect for total bacterial count. In Ireland, Berry et al. (20) reported higher SCC and total bacterial counts during fall and winter and lower counts during spring. They related their findings to the seasonal calving system in Ireland. Soler and Ponsell (27) reported higher total bacterial, psychrotrophic, thermoduric and coliform counts in summer and lower counts in winter. Similar results were observed earlier by Jones et al. (28) who indicated that higher summer temperature may allow the growth of thermoduric and coliform bacteria on milking equipment especially under improper cleaning and sanitation of milking equipment. Our counts were in agreement with the previous results with regard to occurrence of lower counts in winter, however, high counts did not follow consistent pattern for PIC and LPC counts. The different effects of some of the seasons over the 2 years suggest that the effect of season may be influenced by other farm management practices.

2.6 Conclusions

The results of this study provide insight into the current state of microbiological quality of BTM milk in PEI. The majority of samples tested for milk quality parameters were below the regulatory limit of the province (TAC and SCC) or the acceptable limits suggested by the literature (PIC, LPC, and CC). The weak correlation among these parameters indicates differences in on-farm sources for each test and lack of predictive

ability among tests. The study also indicates that seasonal effects may be attributed to the seasonal changes in management practices.

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2.8 References

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Table 2.1 Frequency distributions of total aerobic, preliminary incubation, laboratory pasteurization, coliform and somatic cell counts of bulk tank milk samples in Prince Edward Island, Canada from 2005 to 2007.

Parameter[1]	10%	25%	50%	75%	90%	Mean	G. mean[2]
TAC	1000	2100	4900	13000	34000	12800	5300
PIC	1500	3800	13000	47000	100000	29600	12000
LPC	1	4	12	32	100	87	16
CC	0	1	5	17	54	21	8
SCC	84000	122000	187000	282000	387000	218000	184000

[1] TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count, CC = coliform and SCC = somatic cell count.
[2]Geometric mean

Table 2.2 Comparison of bulk tank milk quality parameters among different studies.

Parameter & Study[1]	Good[2]	Acceptable[2]	Bonus	Median	G.mean[3]
TAC	<5,000	<10,000	<15,000		
Current study	50.5%	71%	80%	4900	5300
NY study[4]	27%	50%	NA	10000	11400
PA study[5]	55%	75%	NA	4100	4300
PIC	<25,000	<50,000	<25,000		
Current study	64.4%	76.6%	64.4%	13000	12000
NY study	29%	45%	NA	62000	81000
PA study	NA	NA	NA	12500	8740
LPC	<100	<200	<100		
Current study	90%	92%	90%	12	16
NY study	44%	60%	NA	120	129
PA study	41%	58%	NA	133	125
CC	<10	<50	<25		
Current study	66.3%	89%	71%	5	8
NY study	30%	39%	NA	24	31
PA study	NA	45%	NA	60	70

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count and CC = coliform.
[2]Thresholds based on guidelines by (Murphy, 1997; Ruegg and Reinemann, 2002).
[3]Geometric mean.
[4]NY study: New York study by Boor et al. (1998).
[5]PA study: Pennsylvania study by Jayarao et al. (2004).

Table 2.3 Percentages of herds with tests over the threshold for each of the bacterial counts and somatic cell count.

Percentage of tests over threshold	TAC[1] (>20,000)	PIC[1] (>50,000)	LPC[1] (>200)	CC[1] (>50)	SCC[1] (>400,000)
0	2.54	1.27	23.31	16.10	28.03
>0 & <0.05	8.90	3.39	34.32	32.20	30.13
≥0.05 & <0.10	21.19	12.29	16.53	16.53	15.48
≥0.10 & <0.20	32.63	30.93	15.25	19.07	10.88
≥0.20 & <0.30	24.15	24.58	4.66	8.90	7.11
≥0.30 & <0.40	7.63	11.44	2.97	2.97	4.60
≥0.40	2.97	16.10	2.97	4.24	3.77

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count, CC = coliform and SCC = somatic cell count.

Table 2.4 Spearman's rank correlation among total aerobic, preliminary incubation, laboratory pasteurization, coliform and somatic cell counts of bulk tank milk samples in Prince Edward Island, Canada.

Parameters[1]	TAC	PIC	LPC	CC	SCC
TAC	1.000				
PIC	0.574	1.000			
LPC	0.158	0.130	1.000		
CC	0.217	0.200	0.158	1.000	
SCC	0.165	0.085	0.253	0.157	1.000

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count, CC = coliform and SCC = somatic cell count.

Table 2.5 The percentiles of the coefficient of variation (CV) for different milk quality parameters.

Parameter[1]	Mean[2]	25%	50%	75%
TAC	0.15	0.12	0.14	0.17
PIC	0.16	0.13	0.15	0.18
LPC	0.42	0.32	0.42	0.49
CC	0.50	0.42	0.50	0.58
SCC	0.03	0.02	0.03	0.04

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count and CC = coliform, SCC = somatic cell count.
[2]Mean coefficient of variation of all herds.

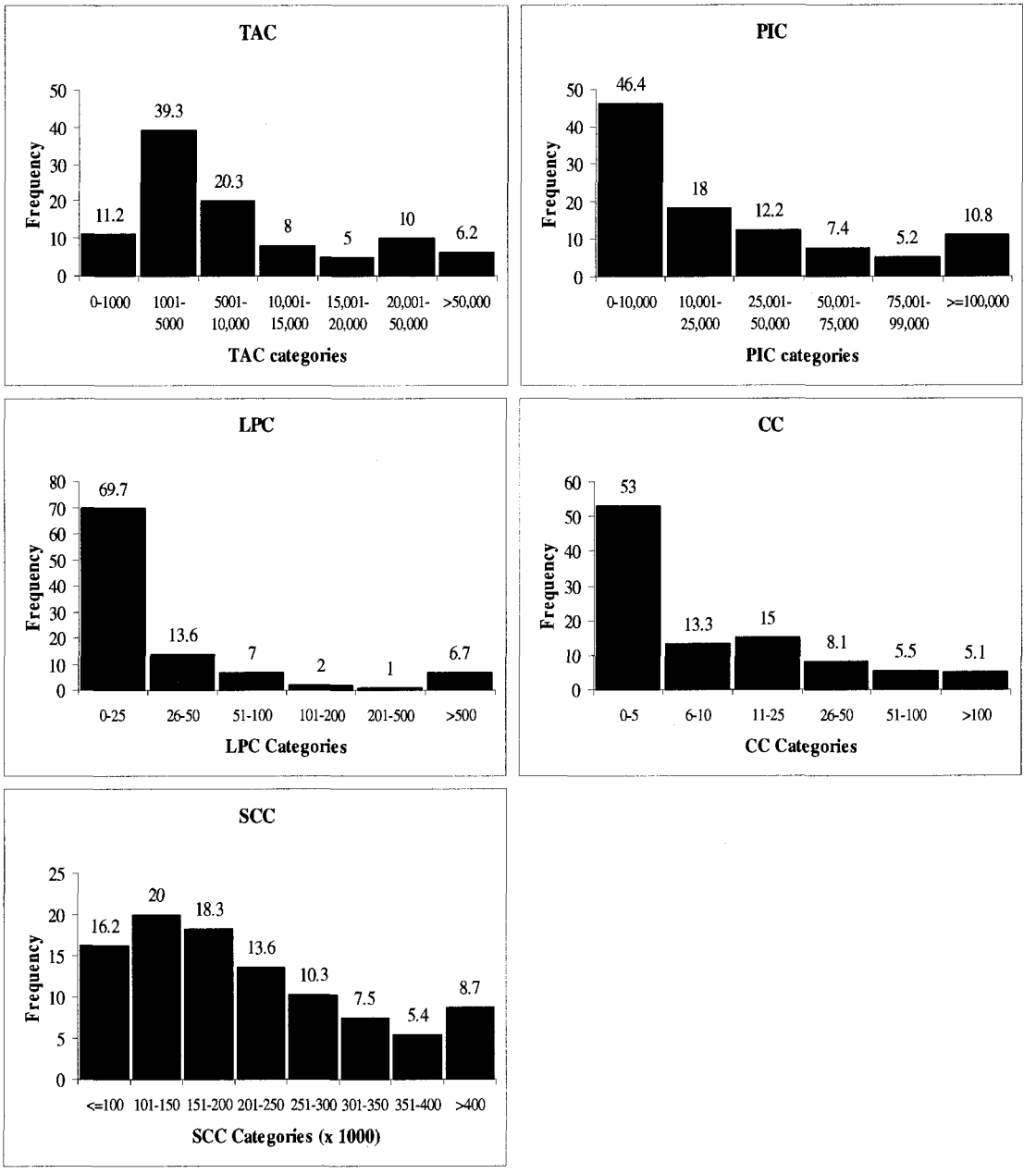

Figure 2.1 Frequency distributions of total aerobic count (TAC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), coliform count (CC) and somatic cell count (SCC) of bulk tank milk samples in Prince Edward Island, Canada. Percentages are displayed at the top of each interval.

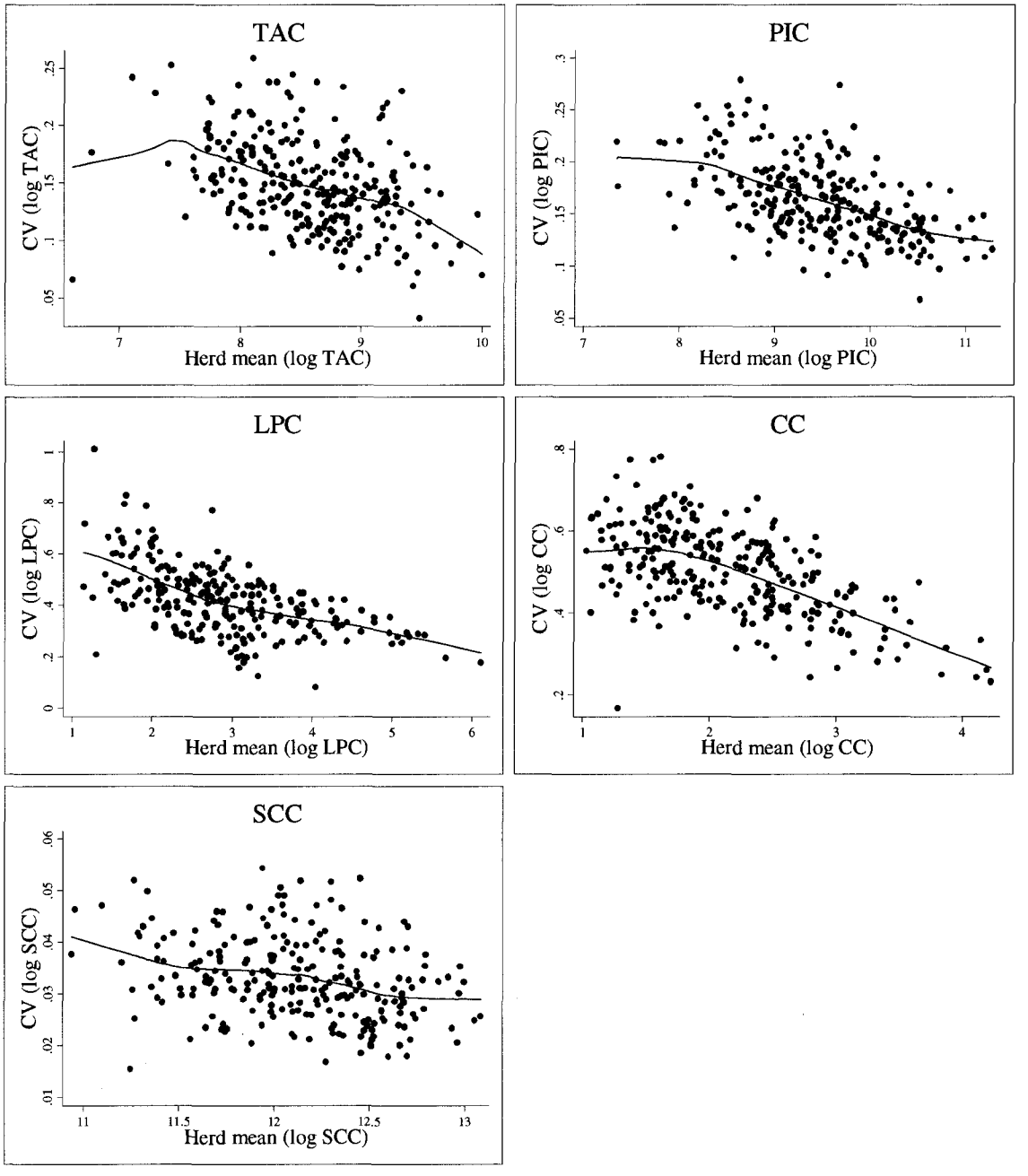

Figure 2.2 The relationship between coefficient of variation and mean herd count for total aerobic count (TAC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), coliform (CC) and somatic cell count (SCC).

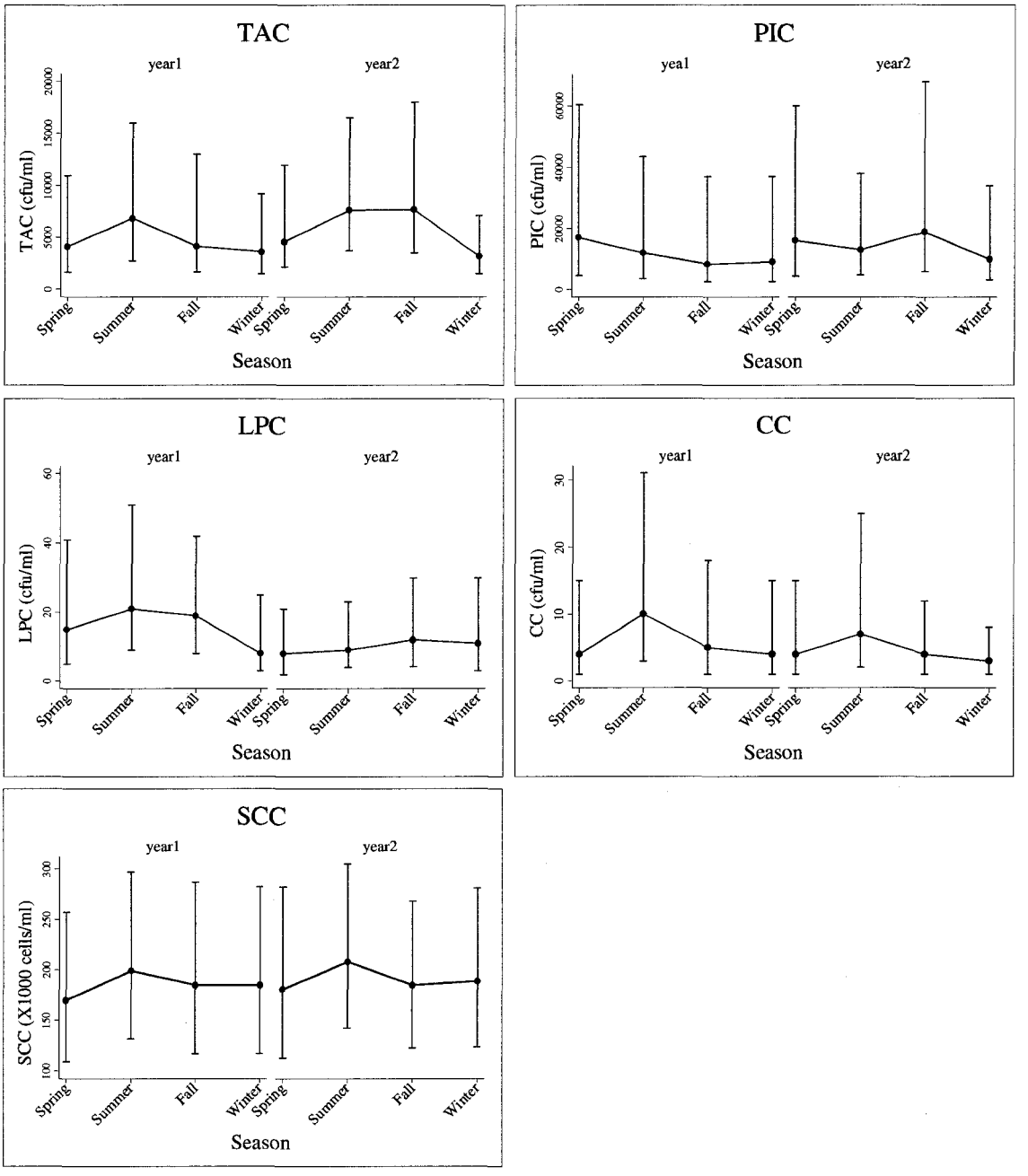

Figure 2.3 The median, first and third quartile of total aerobic count (TAC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), coliform count (CC) and somatic cell count (SCC) of bulk tank milk samples for each season by study year.

CHAPTER 3. THE ASSOCIATION BETWEEN BULK TANK MILK ANALYSIS FOR RAW MILK QUALITY AND ON-FARM MANAGEMENT PRACTICES

3.1 Abstract

Our objective was to determine the risk factors associated with bacteriological quality of bulk tank milk. Bulk tank milk samples were collected from all Prince Edward Island dairy herds (n=235) from March 2005 to March 2007. Biweekly total bacterial, preliminary incubation, laboratory pasteurization, and coliform counts were conducted using a Petrifilm culture system. Data for on-farm risk factors were collected via a mail-out survey which consisted of 4 main sections: 1) general farm demographics and management, 2) cow cleanliness and hygiene, 3) milking procedures and mastitis control, and 4) equipment maintenance and cleaning.

Both total aerobic and preliminary incubation counts were positively associated with the amount of soiling on the teats prior to udder preparation, manual cleaning of the bulk tank, and the use of a certain type of detergent. Additionally, various methods of premilking udder preparation were important, with pre-dip followed by drying being superior to other methods in reducing the bacterial counts. The laboratory pasteurization count was positively associated with the presence of a plate cooler and inadequate frequency of acid wash, whereas having a water purification system was protective. Finally, for coliform count, clipping udder hair and automated washing of the bulk tank were protective, whereas increasing herd size and inadequate frequency of acid wash were risk factors. Season was a significant predictor for all bacterial counts with the lowest counts tending to occur in winter.

3.2 Introduction

High quality raw milk is important for production of high quality pasteurized milk and dairy products. The production of milk with low bacterial counts starts at the farm and is influenced by many procedures related to on-farm management practices. At the farm level, microbial contamination of bulk tank milk (BTM) occurs via 3 main sources: bacterial contamination from the external surface of the udder and teats, from the surface of the milking equipment, and from mastitis organisms from within the udder (1).

The levels and types of microorganisms in BTM provide information on the hygienic conditions during various steps of milk production at the farm. A number of tests are used to monitor hygienic quality of raw milk including: total aerobic count (TAC) which is an alternative to standard plate count (SPC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), coliform count (CC), and speciation of bulk tank milk for mastitis-causing microorganisms (2).

The TAC is the most common method for evaluation of bacterial quality of raw milk. This count estimates the total number of bacteria present in raw milk at the time of pick up from the farm. It provides an overall measure of hygienic quality of milk, however it has a limited diagnostic value in identifying the source of bacterial contamination. The PIC is a selective test measuring psychrotrophic bacteria which grow and multiply under improper refrigeration conditions. These organisms can create undesirable odors and off-flavors. Many psychrotrophic bacteria can also produce heat-stable enzymes which survive pasteurization and cause degradation and reduction in the shelf-life of pasteurized milk and milk products (3).

The LPC is another selective test which estimates the number of thermoduric bacteria which survive a laboratory-scale batch pasteurization process. Thermoduric bacteria have been associated with spoilage of pasteurized milk. Thermoduric organisms come mainly from the surfaces of poorly-cleaned farm equipment. The CC measures the number of coliform bacteria in milk. These organisms primarily originate from the cow's environment, and elevated counts indicate unsanitary production practices. Coliforms can also incubate on residual films of improperly cleaned milking equipment (4).

Although it is well recognized that good quality raw milk is essential for producing quality milk and milk products, there is limited information available on the influence of various management factors on bulk tank bacterial counts. Therefore the objective of this study was to assess the relationships between herd management practices and bacterial levels that characterize the hygienic quality of raw milk.

3.3 Materials and Methods

3.3.1 Data collection

***Milk sampling*:** Bulk tank raw milk was collected from all Prince Edward Island dairy herds (n = 235) every other week by licensed milk haulers over a two-year period (March 2005 to March 2007). Samples were collected in 30 mL sterile screw cap tubes (Starplex Scientific Inc., Etobicoke, Ont.) and held on ice until arrival at the laboratory. All microbiological analyses were performed within 36 h of pick-up at the farm.

On-farm data collection: For collecting data on risk factors, a questionnaire was designed with closed questions only. The questionnaire was comprised of 4 main sections (general farm demographics and management, cow cleanliness and hygiene, milking

procedures and mastitis control, and equipment maintenance and cleaning) with 6 pages and 50 variables, and was pre-tested on 3 farm owners for clarity and ease of administration. The questionnaires were revised where necessary. The final version of the questionnaire was sent to all dairy farms in PEI in October 2005, with telephone follow-up for all non-responders. A copy of the questionnaire can be found in Appendix A.

3.3.2 Bacteriological analysis of BTM

Bulk tank milk samples were examined for TAC, PIC, LPC, and CC using the Petrifilm culture system (3M Canada, London Ontario). The TAC, LPC and CC were conducted according to Standard Methods for the Examination of Dairy Products (SMEDP) procedures (5). The PIC was performed as described by Richardson (6). For TAC and PIC, 1/100 dilution of milk was used. For LPC and CC, one ml of milk was cultured directly on Petrifilm. Plates for enumeration of TAC, PIC, and LPC were incubated at 32 ^{o}C for 48 h. Plates for CC were incubated at 32 ^{o}C for 24 h. All plates were read using an automated counter (3M Petrifilm Plate Reader, 3M Canada, London Ontario), and data were stored in Excel spreadsheets (Microsoft, Seattle, WA). The maximum reading by the automated reader is "> 999", which corresponds to a minimal bacterial load of 100,000 in the case of TAC and PIC, and 1,000 in the case of LPC and CC. All plate counts were expressed as the number of colony-forming unit per milliliter (cfu/ml). Additional information on bulk tank milk analyses and descriptive statistics for each test can be found in Chapter 2.

3.3.3 Statistical analysis

Data Manipulation: Data from questionnaires were coded and entered twice with data-entry software (EpiData Entry; Lauritsen and Bruus, 2006), and both entries were

compared, to check for errors. Both laboratory and on-farm data were merged into a single database using Stata version 10 (Stata Corp, College Station TX, USA). A new variable representing premilking udder preparation was created from combinations of the variables: dry wipe, teat wash, pre-dip, and udder drying.

Simulation approach for censored data: TAC values recorded as 100,000 were right censored because the microbiological procedure did not allow recording of values above 999. To deal with the censoring, we computed artificial values >=100,000 to replace the censored values. The prediction was based on a linear mixed model for log-transformed TAC values with herd random effects. For each right censored value, the predicted value from the model and the estimated within-herd standard deviation were taken as parameters of a normal distribution from which a simulated observation was drawn, conditional on being greater than 100,000. This approach may be described as imputation by draws from a predictive distribution (7). The primary purpose of the imputation was to enable data analysis by standard linear mixed model software, in order to facilitate variable selection and model validation. As a sensitivity analysis, multiple sets of imputed values were generated and the results of the corresponding linear mixed models compared. The same approach was used for right censoring of PIC values at 100,000.

Multivariable associations: Association between management practices and each of the TAC and PIC in bulk tank milk was examined using a linear mixed model with herd random effects and autoregressive correlation structure for the repeated measures on herds (PROC MIXED; SAS software version 9.1; SAS Institute, Inc., Cary, NC). To approximate the normal distribution, a natural logarithmic transformation of the TAC and PIC was used.

The LPC and CC had bimodal distributions, so the variables were dichotomized and analyzed using generalized estimating equations (GEE) with binomial distribution, a logit link, and an autoregressive correlation for repeated measures on herds. For variance estimation, the Huber-White/sandwich estimator of variance was used. The thresholds used for categorizing LPC and CC were greater than 200 and 50 cfu/ml, respectively. These thresholds were selected based on previous literature (1).

Potential risk factors for each of the outcomes were initially screened using unconditional associations ($P < 0.15$). Subsequently, multivariable analysis was conducted and predictors with $P < 0.05$ were retained in the final model. Two-way interactions among all predictors that were significant in the final main effect model were evaluated. Observations that did not fit the model were also examined using standardized residuals for TAC and PIC models (8).

3.4 Results

3.4.1 Descriptive statistics

Over the 2 year study period, approximately 11,099 bulk tank milk samples were evaluated for each of TAC, PIC, LPC, and CC, resulting in 44,396 individual tests. The mean and median values were 12,800 and 4,900 cfu/ml for TAC, 29,600 and 13,000 cfu/ml for PIC, 87 and 12 cfu/ml for LPC, and 21 and 5 cfu/ml for CC, respectively. There was moderate correlation (0.58) between TAC and PIC. All other correlation coefficients among laboratory outcomes were low ≤ 0.23 (See, Chapter 2). Of 235 producers, 153 completed the mail out survey giving a response rate of 65%. There were no significant differences between the mean counts between responders and non-responders for each of TAC, PIC, and CC (Table 3.1). The mean LPC was significantly

higher ($P = 0.002$) for non-responders, however the mean count in both groups was lower than the threshold for high LPC.

3.4.2 Unconditional associations

The unconditional associations ($P < 0.15$) between management factors and each of TAC and PIC are reported in Table 3.2. In addition to the variables retained in the final model, both TAC and PIC were positively associated with the following premilking udder preparation procedures: dry wiping of all teats, not using pre-dip, and using the same towel for udder drying or not drying the udder at all. On the other hand, udder hair clipping, and using detergents, acids, and sanitizers at higher frequency (twice vs. once or less/day) were protective.

Table 3.3 shows the variables that were associated with LPC and CC in BTM. In addition to variables retained in the final model, LPC was positively associated with the use of free stall as opposed to tie stall. The risk of elevated CC was positively associated with increasing herd size, using a milk parlor or a bucket milking system as opposed to a pipeline, cows being confined during summer compared to pastured cows, and using the same towel for udder drying as opposed to single use paper towel.

3.4.3 Multivariable models for TAC and PIC

Table 3.4 shows the risk factors for elevated TAC. The mean log TAC was positively associated with the presence of >10% of cows with dirty teats prior to udder preparation in winter and with using a dry wipe or water to wash the teats versus the use of pre-dip. Using a commercial disinfectant towel alone (without subsequent drying) was also associated with elevated TAC. Additionally, manual cleaning of the bulk tank and use of a certain type of detergent were also associated with high TAC. Season was

strongly associated with TAC, where the association was positive during summer and negative during winter. However, the effect of fall season was not consistent over the two years. There was no significant difference between fall and spring in year one, whereas in year two fall was a risk factor.

The distribution of the variances in the final TAC model indicates that most of the variation was attributed to within-herd variances and the intra-class correlation (ICC) was 0.09 (correlation between 2 observations within the same herd).

The PIC model produced the same set of risk factors as for TAC with some changes in the magnitude of the coefficients and the level of significance (Table 3.5). Additionally, the seasonal effect for PIC was different. In year one, all seasons were associated with lower PIC count compared to spring, whereas in year two, fall was associated with elevated PIC. For the PIC model, the ICC was 0.13.

For both TAC and PIC models, there was no evidence of non-normality or heterogeneity in the distribution of the residuals. Both TAC and PIC models were refit using multiple sets of imputed values and all models produced very similar results (data not shown).

3.4.4 Generalized Estimating Equations for LPC and CC

The GEE results for LPC are presented in Table 3.6. The risk of having high LPC (>200 cfu/ml) was associated with having a plate cooler and with inadequate frequency of acid wash, whereas having a water purification system was protective. There was no significant interaction between year and season. The LPC was highest during summer and lowest during winter. The within herd autocorrelation for LPC was 0.20.

The GEE results for CC are shown in Table 3.7. Udder hair clipping and automated cleaning of the bulk tank were protective, whereas herd size and inadequate frequency of acid wash were risk factors. The CC was highest during summer and lowest during winter. The within herd autocorrelation for CC was 0.30.

3.5 Discussion

3.5.1 Factors associated with TAC and PIC

The risk factors for elevated TAC and PIC were very similar. The amount of dirt on the teats prior to pre-milking udder preparation was positively associated with both TAC and PIC. Dirty udders and teats are considered important sources of environmental bacteria in milk (9). As the proportion of cows with dirty udders and teats increased, the time required for premilking udder preparation will presumably also increase which may have influenced milking efficiency and may have led to inadequate preparation of the udders and teats. Previous studies have reported a positive association between the degree of udder contamination and the level of mastitis as measured by individual cow linear score (10-12). Additionally, Schreiner and Ruegg (10) reported that dirty cows were 1.5 times more likely to be infected with a major mastitis pathogen than clean cows. Previous research by our group also found a positive association between udder hygiene score and bacterial counts in BTM (13).

Effective premilking udder hygiene is important for the production of high quality milk and the control of mastitis. The objective of premilking udder preparation is to milk clean and dry teats (9, 14). Premilking teat disinfection has been associated with reduction in SPC and CC (9, 14), SPC and PIC (2, 13), and total bacteria and anaerobic

spore counts (15, 16). On the other hand, Gibson et al. (17) reported no association between premilking teat-cleaning regime and total bacterial, Enterobacteriaceae, and *E. coli* levels in milk.

In this study, pre-dipping followed by drying the teats with single-use towel was associated with the lowest bacterial counts compared to other methods of teat preparation. Pre-dipping the teats with approved disinfectant is considered the most effective way of teat disinfection and drying of the teats before milking is considered the most important step in a teat cleaning regime (18).

Using water to wash the teats without drying was associated with elevated TAC and PIC. Water laden with bacteria on the udder and teat surfaces can enter the teat cup liners and increase bacterial contamination of milk (19). Higher bacterial counts were observed when the same towel was used for drying multiple cows after washing compared to when a single towel was used for each cow. Sharing the same towel between cows increases the risk of transmission of mastitis pathogens among animals and reduces the efficiency of drying of the teats.

The efficiency of a commercial disinfectant towel in reducing TAC and PIC was related to the method of use. When used alone, it was associated with the highest bacterial counts. However, when followed by drying, their effect was not different from pre-dipping and drying. These results indicate that the use of a medicated towel alone does not adequately kill and remove bacteria from the teats. Additionally, these results indicate that manual drying of the teats is an important step for reducing bacterial burden of the teats. The effect of manual drying may be related to physical action on the teat surface and scrubbing of the teat ends (15).

The results related to premilking udder preparation highlight the importance of chemical sanitization and udder drying in premilking teat cleaning effectiveness, as has been reported by others (9, 16).

Manual cleaning of the bulk tank was also associated with an increased risk of elevated TAC and PIC levels. Manual cleaning of the bulk tank was associated with a lower frequency of detergent and acid use. In another study, manual cleaning was also associated with lower temperature of the cleaning solution (13). The use of a certain type of detergent was also a risk compared to other types. This detergent was a powdered chlorinated detergent available in bulk. The reason for reduced efficiency may be related to improper storage of the product which allows the loss of chlorine during storage.

The results showed strong seasonal variations in TAC and PIC, with higher TAC in the summer, whereas PIC was elevated in the spring. On the other hand, both counts tended to be lower during winter. High TAC during summer months has been reported previously (20, 21). Additionally, low TAC and PIC during winter were reported by Soler and Ponsell (20). Higher counts during summer and spring may be related to warmer ambient temperature allowing bacteria to grow faster. Interestingly, the effect of fall was not consistent over the two years for both TAC and PIC, with the second fall being associated with high risk of elevated counts. There was no substantive variation in ambient temperature and precipitation (data not shown) between the 2 fall seasons. This suggests that variations in seasonal data could be influenced by other management practices such as environment and cow hygiene.

The low values of the ICC suggest that herd evaluation should rely on several measurements of each test and not on a single value.

3.5.2 Factors associated with LPC and CC

Farms without a water purification system were 5.5 times more likely to have elevated LPC than farms that did. Water purification devices remove bacterial contaminants from water. Contaminated water could be a source of *Pseudomonas* spp., coliform, and other Gram-negative bacteria (22). Despite the fact that the effect is highly significant, only 4 herds had a water purification system, so this result should be interpreted with caution.

The presence of a plate cooler was associated with an increased risk of elevated LPC. The fluid dynamics and large surface area can cause debris to accumulate in the plate cooler. The presence of debris together with the difficulty associated with the cleaning of this part of the milking equipment may lead to bacterial film development.

Inadequate frequency of acid wash was a risk factor for high LPC. Acid wash is important for dissolving inorganic mineral deposits (4). Inadequate acid wash frequency may allow precipitation of minerals on the surface of milking equipment which subsequently allows bacterial attachment and formation of biofilms.

Seasonal variations were also evident for LPC, with summer being a risk factor, whereas winter was protective. These results agree with previous finding by Jones et al. (23) and Soler and Ponsell (20). They indicated that higher summer temperatures may allow the growth of thermoduric and coliform bacteria on milking equipment, especially under conditions of improper cleaning and sanitation of milking equipment.

The CC was associated with 5 predictors. Being a herd with larger size was a risk factor for elevated CC. The causal pathway between herd size and CC level is not known.

However, this association could be attributed to other management factors that are highly correlated with herd size such as type of milking system and whether the cows were confined or went to the pasture. Larger size herds tend to be confined indoors which exposes the udder and teats to greater contamination (24).

Udder hair clipping was protective. This finding agrees with previous work (25). Clipping udder hair will reduce the amount of dirt that may attach to the udder and teats. Vissers et al. (26) indicated that the concentration of microorganisms transmitted to milk via dirty teats depended on the amount of dirt and the concentration of microorganisms in this dirt. Subsequently reducing the amount of dirt on the teats will reduce the risk of microbial contamination of milk.

Manual cleaning of bulk tank was also a risk for elevated CC. Other factors, including frequency of acid rinse and seasonal variations, were similar to LPC.

For LPC and CC, the amount of variability and magnitude of high counts was relatively small, thus reducing the ability to identify risk factors for LPC and CC.

3.6 Conclusion

This study highlights the importance of using several bacterial counts (TAC, PIC, LPC, and CC) as indicators of on-farm hygienic conditions during milk production. The TAC and PIC were mainly associated with the proportion of cows soiled with manure, method of premilking udder preparation, and manual cleaning of the bulk tank. The LPC was positively associated with not using a water purification system, use of a plate cooler, and infrequent acid wash. A low CC level was related to udder hair clipping, automated cleaning of the bulk tank and using acid wash twice a day. Season was a significant predictor for all counts, with lower counts tending to occur in winter. The within herd

autocorrelation was weak for all counts, suggesting that herd evaluation can not rely on a single observation.

3.7 Acknowledgements

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Table 3.1 Comparison of the natural logarithmic mean herd counts between responders and non-responders.

Parameter[1]	Responders (n=153)	Non-responders (n=82)
TAC	9.32[a] (11,200)[2]	9.35[a] (11,500)
PIC	10.14[b] (25,300)	10.26[b] (28,600)
LPC	3.69[c] (40)	4.19[d] (66)
CC	2.55[e] (13)	2.74[e] (15)

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count and CC = coliform.
[2]Back transformed values are shown in parentheses
[a,b,c,d,e] superscripts with the same letter within each row are not significantly different at $P < 0.05$.

Table 3.2 Variables associated ($P < 0.15$) with total aerobic and preliminary incubation counts in bulk tank milk of 153 dairy herds in Prince Edward Island.

Variable	Percent	TAC [1]		PIC [1]	
		Estimate	*P*	Estimate	*P*
Stall base			0.10[2]		
Concrete	33	-	-		
Mattress	27	-0.23	0.03		
Rubber	30	-0.09	0.37		
Clay	10	-0.25	0.08		
Frequency of bedding change					
Once or more/day	82				
One or Less every 2 days	14	0.24	0.05	0.29	0.10
Less than two/week [3]	4				
Udder hair clipping					
Yes vs. no	70	-0.21	0.02	-0.28	0.02
Cows with manure on teats prior to udder preparation in winter			0.10[2]		0.07[2]
< 5%	50	-	-	-	-
5-10%	31	0.10	0.28	0.15	0.26
>10%	19	0.23	0.03	0.36	0.02
Pre-milking udder preparation					
Dry wipe			0.07[2]		<0.01[2]
All teats	35	-	-	-	-
Dirty teats only	8	-0.32	0.06	-0.39	0.08
None	57	-0.17	0.06	-0.36	<0.01
Teat wash			0.04[2]		
All teats	37	-	-		
Dirty teats only	8	-0.01	0.93		
None	55	-0.22	0.01		
Pre-dip			<0.01[2]		0.03[2]
Teat dipper	43	-	-	-	-
Teat sprayer [3]	4				
Commercial disinfectant towel	9	0.28	0.07	0.19	0.36
None	44	0.36	<0.01	0.32	0.01
Udder drying			<0.01[2]		<0.01[2]
Single paper towel	67	-	-	-	-
Single use cloth	14	0.05	0.70	0.13	0.45
Multi-use towel	10	0.37	<0.01	0.44	0.02
Not used	9	0.55	<0.01	0.61	<0.01
Bulk tank cleaning					
Manual vs. automated	13	0.36	<0.01	0.49	<0.01
Frequency of pipeline detergent use					
Twice or more/day vs. once/day	95	-0.34	0.08	-0.50	0.07
Frequency of pipeline acid use					
Twice or more/day	92	-	-	-	-
Once/day	5	0.56	<0.01	0.82	<0.01
Other [3]	3				
Frequency of pipeline sanitizer use			0.06[2]		0.03[2]
Twice or more/day	67	-	-	-	-
Once/day	10	0.28	0.05	0.47	0.01
Once/week	5	0.32	0.12	0.30	.028
Frequency of bulk tank detergent use					
Each pickup vs. every second pickup	91	-0.31	0.03	-0.40	0.03

[1] TAC= total aerobic count, PIC= preliminary incubation count.

[2] The overall P-value for variables with multiple categories.

[3] Categories with less than 5% of observations were not included in the analyses.

Table 3.3 Variables associated ($P < 0.15$) with coliforms and laboratory pasteurization counts in bulk tank milk of 153 dairy herds in Prince Edward Island.

Variable	Percent	CC [1]		LPC [1]	
		OR	*P*	OR	*P*
Herd size [2]		1.01	<0.01	1.002	0.13
Lactating cow house					0.11[3]
Tie stall	60			-	-
Free stall	27			1.42	0.15
Straw pack	13			0.61	0.24
Milking system			0.03[3]		
Pipeline	66	-	-		
Parlor	29	1.51	0.08		
Bucket	5	2.63	0.03		
Cows outside in summer			0.05[3]		
Pasture	74	-	-		
Exercise yard	13	1.15	0.58		
Confined	13	2.11	0.01		
Udder hair clipping					
Yes vs. no	70	0.61	0.04		
Udder drying			<0.01[3]		
Single paper towel	67	-	-		
Single use cloth	14	0.96	0.87		
Multi-use towel	10	2.61	<0.01		
Not used	9	1.27	0.55		
Milking mastitis cow last					
Yes vs. no	83	0.63	0.10		
Proportion of dry cow treatment[2]		0.99	0.12		
Bulk tank cleaning					
Manual vs. automated	13	1.75	0.05		
Water purification system					
Yes vs. no	3			0.29	<0.01
Water softener					
Yes vs. no	15			0.61	0.07
Precooler					
Yes vs. no	46			1.65	0.02
Frequency of acid wash					
Once or less vs. twice/day	12	2.19	0.01	2.27	<0.01

[1] CC= coliform count, LPC= laboratory pasteurization count.
[2] Continuous predictors
[3] The overall P-value for variables with multiple categories.

Table 3.4 Linear mixed model of risk factors associated with the mean natural log total aerobic count in raw milk based on 153 dairy herds in Prince Edward Island.

Variables	Estimate	S.E.	*P*
Fixed part of the model			
Intercept	8.63	0.15	<0.01
Percent of manure on the teat prior to udder prep. in winter			0.04[1]
< 5%	-	-	-
5-10%	0.06	0.09	0.45
>10%	0.26	0.10	0.01
Pre-milking udder preparation			<0.01[1]
Pre-dip and drying	-	-	-
Commercial towel and drying	0.07	0.18	0.69
Wash and dry with single towel/ no dry	0.32	0.09	<0.01
Wash and dry with multiple towel	0.51	0.19	<0.01
Dry wipe with single or multitowel	0.54	0.14	<0.01
Commercial towel, no drying	0.79	0.22	<0.01
Bulk tank cleaning			
Manual vs. automated	0.24	0.11	0.03
Pipeline detergent			
Detergent2 vs. others	0.40	0.11	<0.01
Study year			
Year two vs. year one	0.18	0.06	<0.01
Season			<0.01[1]
Spring	-	-	-
Summer	0.33	0.06	<0.01
Fall	-0.01	0.07	0.89
Winter	-0.12	0.07	0.08
Season*year			<0.01[1]
Summer*year2	0.06	0.09	0.53
Fall*year2	0.51	0.09	<0.01
Winter*year2	-0.42	0.09	<0.01
Random part of the model			
Intercept	0.17	0.02	<0.01
Residual	1.74	0.03	<0.01

[1] The overall P-value for variables with multiple categories.

Table 3.5 Linear mixed model of risk factors associated with the mean natural log preliminary incubation count in raw milk based on 153 dairy herds in Prince Edward Island.

Variables	Estimate	S.E.	*P*
Fixed part of the model			
Intercept	10.00	0.23	<0.01
Percent of manure on the teat prior to udder prep. in winter			0.04[1]
< 5%	-	-	-
5-10%	0.11	0.12	0.37
>10%	0.38	0.15	0.01
Pre-milking udder preparation			0.01[1]
Pre-dip and drying	-	-	-
Commercial towel & drying	0.11	0.26	0.66
Wash and dry with single towel/ no dry	0.26	0.13	0.04
Wash and dry with multiple towel	0.53	0.28	0.06
Dry wipe with single or multitowel	0.59	0.20	<0.01
Commercial towel, no drying	0.65	0.31	0.04
Bulk tank cleaning			
Manual vs. automated	0.37	0.16	0.02
Pipeline detergent			
Detergent2 vs. others	0.48	0.19	0.01
Study year			
Year two vs. year one	-0.07	0.08	0.38
Season			<0.01[1]
Spring	-	-	-
Summer	-0.27	0.08	<0.01
Fall	-0.67	0.08	<0.01
Winter	-0.57	0.08	<0.01
Season*year			<0.01[1]
Summer*year2	0.13	0.11	0.23
Fall*year2	1.01	0.11	<0.01
Winter*year2	0.07	0.11	<0.52
Random part of the model			
Intercept	0.38	0.05	<0.01
Residual	2.45	0.04	<0.01

[1] The overall P-value for variables with multiple categories.

Table 3.6 Generalized estimating equations with binary outcome of risk factors associated with laboratory pasteurization count in raw milk based on 153 dairy herds in Prince Edward Island.

Variable	OR	*P*	CI
Water purification system			
Yes vs. no	0.18	<0.01	0.08 – 0.41
Plate cooler			
Yes vs. no	1.68	0.02	1.08 – 2.59
Frequency of acid wash			
Once or less vs. twice/day	2.54	<0.01	1.40 – 2.60
Season		<0.01[1]	
Spring	-	-	-
Summer	1.48	0.03	1.04 – 2.11
Fall	1.11	0.53	0.80 – 1.55
Winter	0.49	<0.01	0.35 – 0.68

[1] The overall P-value for variables with multiple categories.

Table 3.7 Generalized estimating equations with binary outcome of risk factors associated with coliform count in raw milk based on 153 dairy herds in Prince Edward Island

Variable	OR	*P*	CI
Herd size > 50 [1]	2.45	<0.01	1.57 – 3.85
Udder hair clipping			
Yes vs. no	0.67	0.05	0.44 – 0.99
Bulk tank cleaning			
Manual vs. automated	2.93	<0.01	1.57 – 5.47
Frequency of acid wash			
Once or less vs. twice/day	1.43	0.03	1.02– 1.99
Season		<0.01[2]	
Spring	-	-	-
Summer	1.87	<0.01	1.43 – 2.42
Fall	0.93	0.60	0.70 – 1.22
Winter	0.80	0.11	0.61 – 1.05

[1] Herd size was dichotomized due to non linear relation with the log odd of coliform count.
[2] The overall P-value for variables with multiple categories.

CHAPTER 4. RISK FACTORS FOR BACTERIOLOGICAL QUALITY OF BULK TANK MILK IN PRINCE EDWARD ISLAND DAIRY HERDS. PART 1: OVERALL RISK FACTORS

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4.1 Abstract

The objective of this study was to determine on-farm risk factors for bacteriological quality of bulk tank milk using a case-control study design. Bulk tank raw milk quality was evaluated on all Prince Edward Island dairy herds (n = 235) over a two-year period (March 2005 to March 2007). Biweekly total bacterial, preliminary incubation, laboratory pasteurization, and coliform counts were conducted using a Petrifilm culture system. A case-control study was conducted from January 2006 to May 2007. Case and control herds were defined based on the last six analyses of bulk tank bacterial counts prior to on-farm evaluation. Cases were herds which had multiple elevated counts for any of the parameters measured. A total of 69 herds (39 cases and 30 control herds) were evaluated. Data collection included: 1) observations and questionnaire completion on basic hygiene and farm management practices, 2) complete wash analysis of the milking equipment, monitoring the presence of bacterial films on equipment, and evaluation of the cooling system function, and 3) environmental and cow hygiene scoring.

Data were analyzed using multivariable logistic regression. The results of the final model indicated that high alkalinity in the pipeline detergent wash water and poor teat end cleanliness were associated with high bacterial counts in bulk tank milk (OR = 12 and 5.3, respectively). High water temperature of detergent wash and the use of a water softener were associated with low bacterial counts in bulk tank milk (OR = 0.87 and 0.11, respectively). A significant association between udder hair clipping and teat end cleanliness was also observed. In conclusion, this study highlights the importance of udder hygiene and milking system washing factors on hygienic quality of bulk tank milk.

4.2 Introduction

Periodic examination of bulk tank milk (BTM) is useful for monitoring and evaluating raw milk quality produced on a dairy farm. High bacterial counts in raw milk can affect the quality of pasteurized milk and milk products, resulting in lowered shelf-life and reduced consumer acceptance of milk and milk products (1-3). Estimation of the type and number of bacteria in BTM is valuable in understanding and troubleshooting issues related to udder health, milk harvest hygiene, cleaning practices, and milk storage conditions. Tests for milk quality include: total aerobic count (TAC) which is an alternative to standard plate count (SPC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), and coliform count (CC) (4). Among these tests, TAC is the most frequently used in regulatory programs and reflects the general hygienic condition during milk production. Each of the other tests identifies potential contamination sources of concern for milk quality (5).

Microbial contamination of raw milk may occur from three main sources: from within the udder (mastitis-associated organisms), from environmental organism transfer via dirty udder and teat surfaces, and from improperly cleaned and sanitized milking equipment. Additionally, improper cooling and prolonged storage of milk can also influence bacterial counts by increasing the rate of bacterial growth during storage of milk. Because different types of bacteria can contaminate BTM through various and multiple sources, it is not always straightforward to determine the cause of high bacterial counts in milk (5). Accordingly, several guidelines have been developed during the last two decades to facilitate the interpretation of BTM bacterial

counts and to relate these counts to different sources of contamination at the farm (4, 6, 7).

Although it is well recognized that good quality raw milk is essential for producing quality milk and milk products, there is limited information available on the influence of management factors on bacterial counts of BTM. Furthermore, no observational studies have been conducted to evaluate multiple risk factors, and the interactions that might exist between these factors, that could affect the quality of raw milk. The focus of this study was to determine on-farm risk factors that could be associated with elevated BTM bacterial counts using a case-control study design.

4.3 Materials and Methods

4.3.1 Study Design

Bulk tank raw milk was collected from all Prince Edward Island dairy herds (n = 235) every other week by licensed milk haulers over a two year period (March 2005 to March 2007). For each sample, TAC, PIC, LPC, and CC were conducted using Petrifilms (3M Canada, London Ontario). Petrifilms were read electronically, using the Petrifilm Plate Reader (3M Canada, London Ontario) and data were stored in Excel spreadsheets (Microsoft, Seattle, WA) prior to transfer into statistical analysis software.

For the assessment of risk factors, a case-control study was conducted from January 2006 through May 2007. Case and control herds were defined based on the last six analyses of bulk tank bacterial counts prior to on-farm evaluation (approximately three months). To be classified as a case, the herd was required to have at least four high TAC or PIC or CC, or three high LPC measurements out of the last six analyses. Thresholds for high counts were: >20,000 cfu/mL for TAC, >50,000 cfu/mL for PIC,

>200 cfu/mL for LPC, and >50 cfu/mL for CC based on previous literature (4). Control herds had low counts in all of the last six analyses, for all the parameters (TAC, PIC, CC, and LPC) of interest. Every three months throughout the study period, a list of case and control herds was created and herds were selected from these lists using a formal random procedure. Once a farm was selected, it became ineligible for reselection even if its status changed later on in the study period. Herd was the study unit and the target population included all dairy herds in Prince Edward Island, Canada.

4.3.2 On-farm Data Collection

There were three main aspects to the data collection process. The first aspect was observation and recording of data on basic hygiene and management practices in four main areas: 1) general farm demographics and management, 2) cow cleanliness and hygiene, 3) milking procedures and mastitis control, and 4) equipment maintenance and cleaning.

The second aspect was a full evaluation of equipment hygiene and cooling function. This evaluation was carried out by an experienced technologist, trained in equipment analysis. Evaluations of the equipment wash and sanitization cycle were conducted for both the milking system and the bulk tank. This analysis consisted of recording the start and end water temperature (°C) of each cycle (water rinse, alkaline wash, acid rinse). For the alkaline wash cycle, the alkalinity (ppm), chlorine content (ppm), and pH were also recorded. For the acid rinse cycle, the pH was recorded. Finally, a water hardness score in grains per gallon (gpg) was conducted on the water source used for wash procedures. All chemical testing was conducted using LaMotte water quality testing kits (LaMotte Comp., MD, USA).

For evaluation of physical cleaning, cleaning-ball function of the bulk tank was scored by observing the spray distribution during the wash cycle and scored on a scale from 1 (poor) to 5 (excellent). Physical cleaning of the pipeline was evaluated by graphing the vacuum and scoring the bolus (slug) formation on a similar 1 to 5 scale. The presence of organic films on equipment was measured using the ATP bioluminescence technique (Charm LUMinator T and PocketSwab Plus, Charm science Inc, MA, USA): Eight sites were evaluated: five in the milking system (pipeline near and far inlets, receiver jar, diverter valve and cross over pipe) and three in the bulk tank (outlet, agitator paddle and back wall). Pocket swabs® were used to swab the surface of each site according to the instructions of the manufacturer. Additionally, the pipeline near and far inlets and the bulk tank were visually inspected after drying, using a one million foot candle light and were given a score from 1 to 6. The scale for milk surface visual scoring was: 1 (clean and shiny), 2 (<1% film covering), 3 (1-5% film covering), 4 (>5 and <15% film), 5 (15-40% film), and 6 (>40% film covering).

For evaluation of milk cooling system efficiency, milk holding temperatures were recorded electronically every minute for one on-farm storage cycle (approximately two days) using a HOBO U12 temperature data logger (Onset Computer Corp., MA, USA).

The third aspect consisted of environmental and cow hygiene scoring. Based on the number of rows in the stables, four to six milking cow stalls were selected at random for hygiene scoring of the stalls. Additionally, two dry cow stalls and one calving pen were also scored whenever available. Stalls were scored for cleanliness using a four-point scale: 1 (stall clean with dry bedding), 2 (<20% of the stall soiled with urine or manure), 3 (20-40% soiling of the stall), and 4 (>40% soiling of the stall). At the same time,

bedding samples from scored stalls were collected and evaluated for total aerobic and coliform counts. Five bites of bedding from the back of the stall were gathered by hand with sterile gloves into plastic zip bag and kept in cooler until submission to the laboratory.

Cows in the selected stalls were also evaluated for hygiene status using the hygiene scoring card from Pharmacia Animal Health (adapted from hygiene scoring card, School of Veterinary Medicine, University of Wisconsin, Madison). Three areas were scored on a scale from 1 (clean) to 4 (dirty): the udder, the lower leg, and the flank. Additionally, four milking cows were evaluated for teat end cleanliness just before attaching the milking unit using cotton swab, to check the effectiveness of teat sanitation and drying using the 4-point scoring system described by Cook and Reinemann (8).

Each farm assessment took two trained technicians approximately four hours to complete, one investigator was responsible for evaluation of cow and environment hygiene and the other for evaluation of the milking system. Copies of the forms used for on-farm data collection can be found in Appendices B, C, and D.

4.3.3 Statistical Analyses

Data Manipulation: Data from the questionnaire and on-farm evaluation forms were coded and entered twice with data-entry software (EpiData Entry; Lauritsen and Bruus, 2006), and both entries were compared, to check for errors. Both laboratory and on-farm data were merged into a single database using Stata version 10 (Stata Corp, College Station TX, USA).

Predictors that were measured at the cow level (udder, leg, flank, teat end cleanliness, and stall hygiene) were converted to herd level variables by taking the

average score of each category. An average for each of udder, leg, and flank hygiene scores was calculated for milking cows and for combined dry cows and calving pen cows. In the same way, an average stall hygiene score was calculated for each of the milking cow stalls and for combined dry cow stalls and calving pens.

Variables measuring similar management procedures were grouped together by taking their average and their internal consistency was assessed using Cronbach's alpha (9). Cronbach's alpha measures the level of correlation among sets of variables recorded at the same time. For a set of variables to be grouped into a block, alpha should be at least 0.70. Two additional measurements were also calculated during evaluation of the alpha: item rest correlation (IRT), the correlation between an item and the scale that is formed by all other items, and average inter-item correlation (AIIC) of all items.

Unconditional Associations: Unconditional associations between the outcome of interest (being a case with high BTM bacterial counts versus a control with low BTM bacterial counts) and each of the predictors was examined using simple logistic regression. Only predictors showing associations with the outcome of interest at $P \leq 0.15$ were considered for subsequent multivariable analyses.

Multivariable Analyses: Variables that were significant in the univariable analyses were offered into multivariable logistic regression model. Non-significant variables were removed sequentially using backward elimination at $P < 0.05$. Two-way interactions among all predictors that were significant in the final main effect model were evaluated. The fit of the final model was assessed using Hosmer-Lemeshow test. Observations that did not fit or had substantial effect on the model were also examined using standardized residuals, leverage values and delta-betas (10).

4.4 Results

4.4.1 Data Description

A total of 69 herds (39 cases and 30 controls) were evaluated for on-farm risk factors for high bacterial count in bulk tank milk. Table 4.1 shows the main characteristics of the dairy herds that participated in the study by case-control status. Both case and control herds had similar herd size with a mean of 69 cows per herd, including milking and dry cows. Compared to control herds, case herds were less likely to clip udder hair, use pipeline detergent twice a day, have automatic cleaning of bulk tank milk, do a yearly check of milk house water for bacteria, or use a water softener ($P < 0.10$). On the other hand, a higher percentage of case herds used water to wash the teats for pre-milking udder preparation ($P < 0.15$).

A comparison of the factors related to wash analysis of the milking system between case-control herds is shown in Tables 4.2 and 4.3. For wash solution temperature, the main differences between cases and controls were the bulk tank alkaline wash start and ending temperatures, where the proportion of herds that had acceptable or higher water temperature was at least 15% greater in the control group. However, the majority of both cases and controls failed to achieve the recommended temperature. Chemical analyses of the wash solution (Table 4.3) showed that most of case herds had hard water (>6 gpg) and a high concentration of the pipeline alkaline detergent (>500 ppm). Visual inspection of the bulk tank after cleaning and drying indicated that only 11% of case herds and 14% of control herds had a clean and shiny tank, whereas, for pipeline inlets, 21% of cases and 40% of controls were clean and shiny.

There were no differences between cases and controls with regard to different factors related to milk cooling temperature. The overall mean holding temperature was 2.8 vs. 3.1°C and the mean peak temperature after addition of the second milking was 8.5 vs. 9°C in case and control herds, respectively. The mean number of minutes above 6°C during the 2 day pickup cycle was 133 vs. 151 and the mean number of minutes above 8°C was 33 vs. 54 in case and control herds, respectively.

Cow and stall hygiene scores are presented in Table 4.4. Control herds had lower (cleaner) hygiene scores for milking cow stalls, and udder, leg, flank, and teat end cleanliness. The mean natural logarithm of TAC for bedding from milking cow stalls was 18.92 and 18.72 cfu/ml for case and control herds, respectively, and the mean CC was 9.49 and 9.21 cfu/ml for case and control herds, respectively, however, these differences were not significant.

4.4.2 Unconditional Associations

Table 4.5 shows predictors that were unconditionally associated ($P \leq 0.15$) with high or low bacterial counts in BTM. These variables can be divided into 4 main groups: 1) hygiene related factors, 2) variables related to water temperature during the start and end of different washing cycles of pipeline and bulk tank, 3) predictors related to chemistry of the wash solution, and 4) other equipment related factors.

The variables udder, leg and flank hygiene were all significant in the unconditional association and were also highly correlated. Cronbach's alpha for these variables was 0.84 (Table 4.6), therefore an index (cow hygiene score) was created by taking the average of these variables: cow hygiene score= [(udder + leg + flank scores)/3]. Similarly, the variables describing the start and end temperature of bulk tank

and pipeline alkaline detergent wash cycle were significant and highly correlated, their Cronbach's alpha was 0.83 (Table 4.6). As such, an index (detergent wash temperature score) was created: detergent wash temperature score= [(bulk tank alkaline wash fill temperature + bulk tank alkaline wash drain temperature + pipeline alkaline wash start temperature + pipeline alkaline wash end temperature)/4].

4.4.3 Multivariable Association

Multivariable statistical analyses produced a model with four significant ($P < 0.05$) predictors (Table 4.7). Two-way interaction terms between these variables were not significant. There was no evidence of lack of fit of the model as indicated by Hosmer-Lemeshow goodness-of-fit test ($P = 0.75$). According to the final model, the risk of being a case (high bacterial counts in BTM) was minimized when: detergent wash temperature was high, a water softener was used, pipeline alkaline wash alkalinity was moderate, and teat ends were clean.

4.5 Discussion

The production of high quality milk with low bacteriological counts begins at the farm and involves multiple factors related to cow, environment and equipment hygiene. The aim of this study was to identify on-farm management factors that influence microbial contamination of BTM, so that the producers can use these results to improve their milk quality.

4.5.1 Data Quality

This study involved evaluation of a large number of on-farm factors that may influence bacterial quality of BTM. These factors were related to three major areas,

environment, cow, and equipment hygiene. All efforts have been made to ensure high quality of the collected data. All evaluations were done by well-trained and experienced technicians who were blind to the case-control status of the farm. The dairy producers were also blind to the case-control status of their farms and were advised during the on-farm visit to do the procedures as they routinely performed them. If some of the case herds were able to guess their case status, this may have lead to underestimation of the effect of some predictors because producers may have used procedures consistent with industry standard during the farm visit and not their routine methods. The majority of risk factors were recorded using objective rather than subjective methods which ensure fairly accurate classification of exposure status of the farms. Even for factors that were recorded on a subjective scale, they were evaluated by the same blinded investigator. Consequently, any source of misclassification will be expected to be equal among cases and controls, and if this results in any bias, it will likely be towards the null (i.e. underestimation of the effects). Cases were defined as having 3 or more high counts out of the last 6 analyses to represent various degrees of severity of cases and to avoid evaluation of very bad herds only. On the other hand, controls were limited to herds with low bacterial count in all tests in the last 6 analyses to avoid misclassification of cases as controls.

Although, the data were extensive and of good quality, we were only able to evaluate 69 herds due to budget and labor limitations. Because of the relatively small number of herds included in the study, only a limited number of variables that were strongly associated with bulk tank bacterial count could be evaluated in the final model. The absence of a particular variable from the final model may be due to the limited

sample size. Therefore, unconditional associations (Table 4.5) should be considered potential factors of interest for future studies and are therefore discussed in the next section. Additionally, the effect of some variables could not be evaluated due to absence of variation between cases and controls. For example bulk tank milk cooling temperature was relatively consistent between cases and controls for all herds.

4.5.2 Unconditional Associations

Four groups of predictors were identified as having unconditional associations. The first group included factors related to cow management: cow hygiene score (combination of udder, leg and flank), teat end cleanliness score, and udder hair removal. Several studies have identified relationships between cow cleanliness and milk quality as measured by SCC (11-13). For example, using a four point udder hygiene score (clean 1 or 2) and (dirty 3 or 4), Schreiner and Ruegg (11) have shown that SCC and prevalence of intramammary infection were higher for dirty animals. Our study further identified the relationships among cow hygiene, teat end cleanliness score, and bacterial count in BTM, with high hygiene score (dirty) being associated with increased risk of having high bacterial counts in BTM.

Cow cleanliness can also affect the efficiency of cow preparation before milking, where dirty cows can double cow preparation time (14). Cleanliness of the udder and teats can be influenced by a number of factors including: transition from summer grazing to winter housing, with housed cows being dirtier than grazing cows (13), fecal consistency, where increasingly fluid fecal consistency being correlated with dirtier cows, frequency of bedding change and quality of bedding, (15), and stage of lactation (12).

There are few studies on the effect of udder hair clipping on milk quality. Barkema et al. (16) reported that herds with low SCC were practicing udder hair clipping more frequently (84 vs. 62%) than herds with high SCC. This study showed that clipping udder hair is associated with having lower bacterial count in BTM (OR = 0.26). Additionally, there was a significant association between udder hair clipping and teat end cleanliness score ($P = 0.01$), where udder hair clipping was associated with a reduction of 0.35 units on the cleanliness scale (indicating cleaner teats). These results confirm the importance of udder hair clipping in decreasing bacterial contamination of raw milk. Another study by Bartlett et al. (17) reported a weak association between clipping udders and decreased intramammary coliform infection. On the other hand, Silk et al. (18) evaluated the effect of removing udder hair surrounding the teats on milk quality, and found that udder hair removal had no effect on milk quality as measured by bacterial count of milk and on the teat surface. They suggested that the lack of effect of udder hair removal may be related to the use of pre-dipping for udder preparation in the study herds, which may be sufficient to remove teat skin bacteria regardless of udder hair removal.

The other three groups of predictors were all related to cleaning of the milking system. Proper cleaning and sanitation of the milking system is one of the most important aspects of producing high quality raw milk. It involves a combination of thermal, chemical and physical processes. A deficiency in any of these parameters could result in soil build up which provides nutrients for growth and multiplication of bacteria between milking times (19). In this study, high temperature of the rinse water and alkaline detergent wash solution was always associated with having low bacterial count in BTM. Hot water is important for emulsifying milk fat and dispersing milk protein, as well as

increasing the cleaning efficiency of chemicals (19). Therefore, checking the hot water supply for adequate temperature is necessary to ensure proper cleaning temperature.

Water is the most important ingredient in cleaning and sanitizing solutions. The properties of water on the farm can affect the cleaning process and milk quality. Our results showed that herds with medium or high water hardness scores were 2.5 and 4.7 times more likely to have high bacterial counts in BTM than herds with lower hardness scores. Hard water can reduce the effectiveness of cleaning chemicals, and may also lead to formation of films or deposits on the milking system (20). Additionally, less frequent checks of milk house water for bacterial contamination and hardness was also associated with higher risk of having high bacterial counts in BTM. Untreated water could be a source of contamination of milking equipment with *Pseudomonas* spp., coliforms, and other gram-negative bacteria (21). Consequently, regular monitoring of microbiological and chemical properties of water will help in identifying water quality problems and implementing the necessary treatment in the proper time.

The efficiency of cleaning and sanitation of the milking equipment was assessed using an ATP bioluminescence method. The ATP method is fast and simple and can monitor both microbial contamination and milk residues (22).

In this study, bioluminescence readings of the bulk tank outlet and the far inlet of the pipeline were associated with high BTM bacterial counts, however, there was no association for the other six sites. Reinemann and Ruegg (22) indicated that there is considerable variation in the ATP data, and the method must be used carefully and with sufficient number of ATP swabs to obtain meaningful results. Our data support this

conclusion, and indicate that while ATP testing may be helpful in hygiene investigations, they must be used as part of thorough system evaluation.

4.5.3 Multivariable Association

The final model had four significant predictors ($P < 0.05$), with high temperature of the detergent wash and the use of a water softener being protective, whereas, high alkalinity and high (dirty) teat end cleanliness scores were risk factors. Adjusted for other variables in the model, for every decrease of one ^{o}C in detergent wash temperature below the average temperature score (52 ^{o}C), the likelihood of having high bacterial counts in BTM increases by a factor of 1.15. Consequently, a ten ^{o}C decrease will increase the risk by a factor of 4. These results confirm the importance of water temperatures. It also supports the report of Guterbock et al. (23) who noted that the cleaning efficiency doubles with every increase of 10 ^{o}C in temperature. Similarly the risk of having high bacterial counts in BTM will increase by 5.3 for each unit increase in teat end cleanliness scores.

Contrary to our expectation, high alkalinity of the pipeline detergent wash water was associated with increased risk of high bacterial counts (OR = 12). The alkaline detergent wash is important for dissolving milk fats, proteins and carbohydrates and for dispersion and suspension of other soil particles so that they can be removed by mechanical action (19) and prevented from deposition on the milking system. A possible explanation of this finding is that higher alkalinity was the result of increased detergent use by producers in response to high bacterial count problems. Finally, the use of a water softener was associated with decreased risk of having high bacterial counts (OR = 0.11).

The effect of water softener use could be explained through the prevention of water hardness problem and its influence on milk quality.

4.6 Conclusions

The results of this study highlight the importance of udder hygiene (especially, teat end cleanliness at milking), alkalinity and temperature of the alkaline detergent wash solution used for cleaning the milking system, and water quality related factors (water softener) for hygienic quality of BTM. Although, only four predictors remained in the final models, all factors in the unconditional associations should be considered for improvement of bacteriological quality of BTM.

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Table 4.1 Characteristics of 69 dairy herds that participated in a case-control study of on-farm risks for raw milk quality in Prince Edward Island, Canada.

Parameter	Cases (n=39)	Controls (n=30)	*P*-value[1]
	Percent[2]	Percent[2]	
Type of housing			0.478
Tie-stall	56	70	
Free-stall	31	23	
Manure/straw pack	13	7	
Milking system			0.756
Pipeline	62	70	
Parlor	33	27	
Buckets	5	3	
Bedding material			0.179
Straw	74	63	
Wood products	21	37	
Sand	5	0	
Frequency of bedding change			0.551
Twice a day or more vs. lower freq	67	73	
Udder clipping (yes vs. no)	56	83	0.015
Milking procedures			
Strip Yes	36	40	0.727
Prewipe teats Yes	26	37	0.324
Wash Yes	41	23	0.122
Pre-dip Yes	51	67	0.199
Udder drying Yes	87	90	0.717
Frequency of pipeline cleaners use			
Detergent:2/d vs. lower	82	96	0.091
Acid: 2/d vs. lower	88	96	0.236
Sanitizer: 2/d vs. lower	85	94	0.325
Frequency of bulk tank cleaners use			
Detergent: after each pickup vs. other	92	96	0.261
Acid: after each pickup vs. other	94	96	0.733
Sanitizer: after each pickup vs. other	89	93	0.694
Bulk tank cleaning			
Automatic vs. manual	79	93	0.105
Milk house water check			
Bacteria: 1/y vs. longer than 1/y	31	70	0.001
Hardness: 1/y vs. longer than 1/y	31	63	0.007
Water softener: yes vs. no	10	37	0.008

[1]P-value was calculated based on Pearson chi square.

[2]Percentage of herds that apply specific management procedure.

Table 4.2 Bulk tank and pipeline wash analyses (wash solution temperature) of the 69 herds that participated in a case-control study of on-farm risks for raw milk quality.

Parameter	Threshold[1]	Cases (n=39)	Controls (n=30)	*P*-value[2]
		Percent[3]	Percent[3]	
Bulk Tank Wash Analyses				
Pre-wash rinse				
Prewash fill temperature	55 °C	27	41	0.140
Prewash drain temperature	38 °C	22	21	0.927
Chlorinated alkaline wash				
Starting temperature	77 °C	0	17	0.008
Ending temperature	43 °C	31	47	0.177
Pipe line Wash Analyses				
Pre-wash rinse				
Starting temperature	55 °C	0	7	0.102
Ending temperature	38 °C	6	16	0.234
Chlorinated alkaline wash				
Starting temperature	77 °C	0	0	-
Ending temperature	43 °C	31	38	0.537

[1]According to IDF Bulletin (Reinemann et al., 2003).
[2]P-value was calculated based on Pearson chi square.
[3]Percentage of herds that had water temperature ≥ recommended value.

Table 4.3 Bulk tank and pipeline wash analyses (chemistry and physical action) of the 69 herds that participated in a case-control study of on-farm risks for raw milk quality.

Parameter	Cases (n=39)	Controls (n=30)	*P*-value[1]
	Percent[2]	Percent[2]	
Bulk Tank Wash Analyses[3]			
Chlorinated alkaline wash			
Active chlorine level ≥100 ppm	44	59	0.244
Active alkalinity level (ppm)			0.946
<400	31	33	
400-800	46	47	
>800	23	20	
Alkaline wash pH ≥ 11	69	73	0.710
Acid rinse pH < 3.5	97	96	0.881
Pipeline Wash Analyses[3]			
Chlorinated alkaline wash			
Active chlorine level ≥75 ppm	64	72	0.566
Active alkalinity level			0.021
<250	05	13	
250-500	44	67	
>500	51	20	
Alkaline wash pH ≥ 11	92	97	0.442
Acid rinse pH < 3.5	91	89	0.773
Water hardness score (gpg)[4]			0.047
≤6	29	57	
7-9	37	29	
≥10	34	14	
Physical action			
Cleaning ball function score ≥ 4	77	78	0.974
Slug score ≥ 4			
Beginning	21	31	0.322
Middle	26	28	0.857
End	31	38	0.537

[1]P-value was calculated based on Pearson chi square.
[2]Percentage of herds that were ≥ recommended value.
[3]Thresholds for chlorine, alkalinity and Ph were selected according to IDF Bulletin (Reinemann et al., 2003).
[4]gpg: grains per gallon.

Table 4.4 Median and mean stall hygiene, cow hygiene and teat end cleanliness scores for case and control herds in the study of on-farm risks for raw milk quality.

Parameter	Cases (n=39)		Controls (n=30)	
	Median	Mean (SD[1])	Median	Mean (SD)
Milking stall	1.75	1.80 (0.69)	1.75	1.67 (0.55)
Dry stall & calving pen	2.00	2.15 (0.82)	1.42	1.84 (0.61)
Milking cow udder	1.50	1.51 (0.44)	1.33	1.36 (0.30)
Milking cow leg	2.00	2.05 (0.62)	1.75	1.80 (0.51)
Milking cow flank	1.50	1.75 (0.65)	1.29	1.52 (0.50)
Dry & calving cow udder	1.00	1.30 (0.55)	1.00	1.24 (0.39)
Dry & calving cow leg	1.50	1.73 (0.63)	1.83	1.87 (0.66)
Dry & calving cow flank	1.50	1.57 (0.83)	1.58	1.81 (0.81)
Average teat cleanliness	1.44	1.60 (0.61)	1.12	1.27 (0.36)

[1]SD= Standard deviation.

Table 4.5 Unconditional associations ($P \leq 0.15$) between case-control status and on-farm risks for raw milk quality in Prince Edward Island, Canada.

Variables	Odds Ratio	P-value
Hygiene related factors		
Teat wash (no vs. yes)	2.28	0.122
Udder hair removal (no vs. yes)	0.26	0.015
Teat end cleanliness score	4.50	0.007
Cow hygiene score[1]	2.70	0.078
Average udder hygiene	2.52	0.150
Average leg hygiene	2.09	0.095
Average flank hygiene	1.88	0.140
Wash analyses: Temperature		
Bulk tank Prewash fill temperature	0.98	0.060
Bulk tank Prewash drain temperature	0.96	0.036
Pipeline rinse start temperature	0.96	0.103
Detergent wash temperature score[2]	0.90	0.005
Bulk tank alkaline wash fill temperature	0.93	0.014
Bulk tank alkaline wash drain temperature	0.95	0.046
Pipeline alkaline wash start temperature	0.92	0.021
Pipeline alkaline wash end temperature	0.93	0.059
Wash analyses: Chemistry		
Pipeline alkaline wash alkalinity	1.003	0.002
Water hardness ≤ 6 (reference)		0.047
7-9	2.54	0.114
≥10	4.73	0.025
Water softener (no vs. yes)	0.20	0.008
Pipeline det freq (twice vs. once/day)	5.55	0.073
Other milking equipment related factors		
Bulk tank outlet bioluminescence score > 0	2.25	0.104
Pipeline far inlet bioluminescence score > 0	2.62	0.053
Tank cleaning (manual vs. automatic)	0.28	0.092
Milk house water bacterial check: yearly vs. longer	5.25	0.001
Milk house water hardness check: yearly vs. longer	3.90	0.007

[1]Average of udder, leg, and flank scores.
[2]Average of alkaline wash start and end temperatures of bulk tank and pipeline.

Table 4.6 Evaluation of Cronbach's alpha for the predictors describing the same management procedure.

Within block factor	Observations	IRC[1]	AIIC[2]	Alpha
Block 1: cow hygiene score				
Udder	69	0.67	0.70	0.82
Leg	69	0.71	0.64	0.78
Flank	69	0.75	0.60	0.75
Test scale	-	-	0.64	0.84
Block 2:Detergent wash temperature				
Bulk tank alkaline wash fill	69	0.63	0.58	0.81
Bulk tank alkaline wash drain	69	0.66	0.56	0.79
Pipeline alkaline wash start	69	0.75	0.50	0.75
Pipeline alkaline wash end	68	0.63	0.58	0.81
Test scale	-	-	0.56	0.83

[1]IRC: item rest correlation.
[2]AIIC: average inter-item correlation.

Table 4.7 Final multivariable logistic regression model for on-farm risks for raw milk quality in Prince Edward Island, Canada.

Case-control	Odds Ratio	P-value	95% CI
Teat end cleanliness score	5.27	0.027	1.20 – 23.09
Detergent wash temperature score[1]	0.87	0.014	0.77 – 0.97
Water softener (yes vs. no)	0.11	0.013	0.02 – 0.62
Pipeline alkaline wash alkalinity >500 ppm	11.88	0.001	2.66 – 53.18

[1]Average of alkaline wash start and end temperatures of bulk tank and pipeline.

CHAPTER 5. RISK FACTORS FOR BACTERIOLOGICAL QUALITY OF BULK TANK MILK IN PRINCE EDWARD ISLAND DAIRY HERDS. PART 2: BACTERIA COUNT SPECIFIC RISK FACTORS

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5.1 Abstract

A case control study was conducted to identify specific on-farm risk factors that influence bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC), and coliform (CC) counts were used to assess bacteriological quality of bulk tank milk. Four case-control groups were defined based on the last 6 results of each test prior to on-farm evaluation. A herd was classified as a TAC or PIC or CC case, when the herd had at least 4 high tests for TAC or PIC or CC out of the last 6 analyses for each test, respectively. For the LPC case group, a herd was required to have at least 3 high results out of the last 6 analyses. Control groups had low counts in the last 6 analyses, for each test in the corresponding case group (TAC, PIC, CC, and LPC). The number of cases and controls in each group were 16 and 39, 21 and 31, 12 and 50, and 8 and 54, for TAC, PIC, CC, and LPC, respectively. Data collection included observation of basic management practices, full analytical evaluation of equipment hygiene and cooling efficiency, and scoring of cow and environmental hygiene.

The results of study showed that TAC and PIC were mainly associated with cow and stall hygiene, with washing the teats with water, not using teat pre-dip, and dirty teats being risk factors. The LPC and CC were related to equipment hygiene, with high counts being associated with low temperature of the detergent wash water, high water hardness score, and high alkalinity of pipeline alkaline detergent wash water. The findings of this study indicate that TAC, PIC, LPC, and CC counts are of considerable value for identifying practices that could influence milk quality.

5.2 Introduction

The production of high quality milk with low bacteriological counts begins at the farm. It involves multiple factors related to cow health and udder hygiene, hygiene of the milking environment in which the cows are housed and milked, and hygiene of the milking equipment. Microbial contamination of bulk tank milk (BTM) occurs through a variety of sources and by different types of microorganisms. Therefore, using multiple bacterial tests that estimate specific groups of bacteria in BTM could provide a detailed picture on inappropriate practices employed on the farm during collection, handling and storage of BTM (1).

In this study, four bacterial parameters were used to assess milk quality: total aerobic count (TAC), preliminary incubation count (PIC), laboratory pasteurization count (LPC), and coliform count (CC). The TAC is an alternative to the standard plate count (SPC). It estimates the total number of aerobic bacteria in raw milk samples and is an important parameter in regulatory and quality incentive programs in many parts of the world. The TAC indicates the general hygienic conditions during milk production, collection, and storage, therefore, it may be of limited importance in identifying specific sources of contamination (2). The PIC quantifies psychrotrophic bacteria which grow at inadequate refrigeration temperature. Psychrotrophs are generally found in untreated water, soil and vegetation. They are introduced into the milk as a result of contamination of milking equipment or the exterior of the udder and teats from these sources (3).

The LPC estimates the number of thermoduric bacteria which survive laboratory-scale pasteurization procedures similar to batch pasteurization (62.8 ^{o}C for 30 min). The main sources of contamination of milk by thermoduric bacteria are poorly cleaned and

inadequately sanitized udders and equipment (3). The CC enumerates coliform bacteria. Coliforms inhabit the intestinal tract of cows and are commonly found in manure, bedding material, soil and contaminated water. They contaminate raw milk through the exterior of udder and teats and contaminated milking equipment.

Previous studies found low correlations among these bacterial counts (4, 5); Elmoslemany et al., Chapter 2). The low correlations among these parameters indicate that they have different sources. Research data on the specific on-farm risks associated with elevated bacterial counts in BTM are limited, therefore the objective of this study was to evaluate on-farm risk factors for high bacterial counts in BTM, through the use of case-control study design. In the first part of this study, the overall risk factors were addressed for high bacterial counts in BTM as defined by the combination of all four bacterial tests (TAC, PIC, LPC, and CC). This paper focuses on specific risk factors for each of the four bacterial counts individually.

5.3 Materials and Methods

5.3.1 Bulk tank milk analyses, study design and data collection

Details on bulk tank milk analyses, study design and data collection can be found in part one of this study (See, Chapter 4). Additionally, descriptive statistics on different bacterial counts can be found in (Chapter 2). Briefly, Bulk tank raw milk quality was evaluated on all Prince Edward Island dairy herds (n = 235) over a two year period (March 2005 to March 2007). Biweekly TAC, PIC, LPC, and CC, were conducted using Petrifilms (3M Canada, London Ontario).

For the assessment of risk factors, a case-control study was conducted from January 2006 to May 2007. Four case-control groups were defined based on the last six

results of each test prior to on-farm evaluation. To be classified as a TAC or PIC or CC case, the herd was required to have at least four high TAC (>20,000 cfu/ml) or PIC (>50,000 cfu/ml) or CC (>50 cfu/ml) counts out of the last six analyses for each test, respectively. For the LPC case group, a herd was required to have at least three high (>200 cfu/ml) LPC results out of the last six analyses. Thresholds for high counts were selected based on previous literature (3). Control groups had low counts in the last 6 analyses, for each test in the corresponding case group (TAC, PIC, CC, and LPC). The number of cases and controls in each group were 16 and 39, 21 and 31, 12 and 50, and 8 and 54, for TAC, PIC, CC, and LPC, respectively.

Data collection included 3 main aspects: 1) observations and questionnaire recordings on basic hygiene and management practices, milking procedures, and equipment cleaning and maintenance, 2) evaluation of cleaning efficiency of the milking system (thermal, chemical, and physical components of cleaning process), monitoring the presence of organic film deposits on milk contact surfaces, and evaluation of efficiency of the cooling system, and 3) evaluation of environmental and cow hygiene. Complete description of on-farm data collection can be found in part one of the study (See Chapter 4).

5.3.2 Statistical Analyses

Unconditional Associations. Unconditional associations between each of the case-control groups (TAC, PIC, LPC, and CC) and management factors were examined using simple logistic regression. For each outcome, only variables with significance level ($P < 0.15$) were considered for subsequent analysis. Due to the low number of cases in each of the case-control groups, multivariable models were unstable and sensitive to minor

changes in the predictor variables, therefore, we could not fit multivariable models. Accordingly, the relationship between factors that were significant in the unconditional association at $P < 0.05$ and each of the outcomes of interest was further explored using correspondence analysis.

Multiple Correspondence analyses. Multiple Correspondence analyses (MCA) is an exploratory technique that can be used to produce a graphical display of the relationship among a set of categorical variables. The MCA technique is not useful for the confirmation or rejection of hypotheses, but is primarily intended to reveal patterns in the data. The technique defines a measure of distance between any two points, where points are the values (categories) of the discrete variables. The proximity of the points to each other indicates the strength of association. The closer together the points are, the more closely they are associated. The value of the outcome variable can also be presented on the same graph to determine which clusters of predictor variable values are associated with the outcome (6). As only categorical variables can be used in this technique, continuous variables such as cow hygiene score, teat end cleanliness score, and detergent wash temperature and alkalinity were first categorized based on the percentiles of the distribution of the variables or, when available, based on recommended thresholds.

5.4 Results

The results of the unconditional associations (P <0.15) between each of the outcomes of interest and different management factors are shown in Tables 5.1 and 5.2. These variables were classified into 4 different groups as indicated in part one.

Group one (Table 5.1) includes factors related to environment and cow hygiene. The TAC was associated with a large number of hygiene related factors. High hygiene scores (dirty) of stall, cow and teat end were associated with elevated TAC in BTM. Additionally, using water to wash the teats for pre-milking udder preparation was also a risk for high TAC. On the other hand, udder hair clipping and the use of teat pre-dip were protective. A similar level of association was also evident between hygiene related factors and PIC except for udder, leg, and flank hygiene scores, which were not associated with PIC. The CC was only associated with leg hygiene and teat end cleanliness scores, with dirty teats or legs being risk factors.

Groups 2, 3, and 4 are comprised of factors related to equipment hygiene (wash solution temperature, chemistry, and physical cleaning). There was an association between most of the temperature related factors and LPC and CC. In all cases high temperature was protective (Table 5.2).

Chemistry related factors were mainly associated with LPC and CC. High alkalinity of detergent wash was a risk factor for all counts. High chlorine concentration of the detergent wash was protective for LPC and CC, whereas high hardness score was associated with elevated LPC and CC (Table 5.2).

The last group was comprised of physical cleaning and other equipment related factors (Table 5.2). High slug score (sufficient physical cleaning) of the pipeline was protective for both TAC and LPC. Less frequent evaluation of milk house water for bacteria and for hardness were risk factors for all bacterial counts. High bioluminescence at the bulk tank outlet was associated with elevated TAC, PIC, and CC. Finally, the use of a water softener was protective for TAC and PIC.

The categories of the continuous variables used for MCA are shown in Table 5.3. Figures 5.1-5.4 summarize the results of MCA. Figure 5.1 illustrates the risk factors for TAC. A low TAC (control) was closely related to using teat pre-dip, clean cow (udder, leg and flank), clean teat end, and udder hair clipping. On the other hand, a high TAC (case) was corresponded with not using teat pre-dip and high cow hygiene score (dirty cow). The graph also highlights the close correspondence between udder hair clipping and hygienic condition of the teat end and the cow.

A low PIC (control) was closely related to using teat pre-dip and high bulk tank alkaline wash temperature, whereas a high PIC (case) was corresponded with a dirty teat end, and not using teat pre-dip (Figure 5.2).

A low LPC (control) was related to high temperature of the detergent wash, low water hardness and medium level of bulk tank alkaline wash alkalinity, whereas a high LPC (case) was mainly related to the use of low temperature of the detergent wash and high level of bulk tank alkaline wash alkalinity (Figure 5.3).

Figure 5.4 shows that low CC (control) was corresponded with a clean teat end, automatic cleaning of bulk tank milk, low water hardness and high or medium detergent wash temperature. On the other hand, high CC (case) was mainly related to dirty teat ends and low temperature of detergent wash.

5.5 Discussion

In order to achieve high raw milk quality, producers should be aware of the factors that influence contamination of raw milk and how they can be controlled. In this study, variables that had significant unconditional associations with milk quality

parameters were mainly related to cow and equipment hygiene and were classified into 4 groups for ease of discussion.

Group one includes variables measuring environmental and cow hygiene. It was observed that high cow (udder, leg, and flank) hygiene and teat end cleanliness scores (dirty) were associated with increased risk of elevated bacterial counts in BTM (Elmoslemany et al., Chapter 4). The current study showed that cow hygiene score was mainly related to TAC, whereas, teat end cleanliness score was associated with TAC, PIC, and CC. Additionally, there was significant ($P < 0.001$) association between milking stall hygiene and each of cow and teat end cleanliness scores, with cleaner stalls being associated with cleaner cows and teats. A clean and dry cow environment is important in preventing environmental mastitis (7). Previous studies indicated that bacterial counts in bedding are positively correlated with stall cleanliness (8) and as the percentage of dirty stalls increased, the risk of clinical mastitis also increased (9). Additionally, Barkema et al. (10) reported that herds with SCC >250,000 cells/mL had more manure in stalls, cleaned stalls less frequently and used less bedding in stalls. In our study, a dirty stall was associated with a high TAC, which could be caused by many factors including mastitis microorganisms (3). Furthermore, a dirty stall was also associated with an elevated PIC, which could be attributed to contamination from the exterior of the udder and teats by bacteria from the cows' environment (5). Although hygiene score of the stall was associated with both TAC and PIC, we did not find any associations between bacterial counts in bedding and any of the milk quality parameters (See, Chapter 4). This may indicate that hygiene scores of the stalls are better predictors of milk quality than bacterial counts in bedding.

Dirty teats and udders are considered some of the main sources of environmental bacteria in milk. Between milking, the teats and udder often become soiled with manure and bedding materials. If the teats were not thoroughly cleaned and dried before milking, this dirt with the associated microorganisms could be transferred into the milk (2). Contamination from the exterior of the udder and teats can contribute microorganisms from the cow environment, such as streptococci, staphylococci, spore-formers, coliforms and other Gram-negative bacteria, which in turn can elevate TAC, PIC, LPC and CC (3). Our results indicate that dirty teats are a risk factor for elevated TAC, PIC, and CC. High numbers of environmental bacteria in BTM indicate a problem related to environmental and milking hygiene (11).

The influence of dirty cows on bacterial counts depends on the extent of soiling of the teat surface and on pre-milking udder preparation practices. The purpose of pre-milking udder hygiene is to reduce the bacterial contamination on the teats to minimal numbers prior to the attachment of the milking unit (12). Galton et al (13) indicated that the use of pre-dipping resulted in 5 and 6 fold reduction in SPC and CC, respectively, as compared to no udder preparation. Recently, Jayarao et al. (5) reported a reduction in environmental mastitis pathogens and psychrotrophic and thermoduric bacteria with the use of teat pre-dipping. On the other hand, washing the teats with water prior to milking resulted in elevated SPC (14) and increased prevalence of coliform mastitis (7).

Our results corroborate earlier findings with respect to the SPC and PIC, however our findings differed with respect to CC, as our study revealed that there was no association between CC and methods of pre-milking udder preparation. In our study, the lack of association between CC and pre-milking udder preparation procedures could be

attributed to the limited number of CC cases, or may indicate that coliforms had contaminated BTM through routes other than contaminated teats, such as milking equipment or a contaminated water source (15). A lack of correlation between pre-milking teat cleaning regimes and environmental bacteria was also reported by (16, 17) which suggests that teat end contamination may not be the primary source of coliform bacteria in BTM.

The last three groups of predictors were all related to cleaning and sanitation of the milking equipment which illustrates the importance of milking equipment as a source of bacterial contamination of raw milk. Adequate cleaning and disinfection of milking equipment is necessary to remove residues and microorganisms from equipment surfaces (18).

In this study, factors measuring the temperature of the wash solution were mainly associated with LPC and CC, with higher temperature being protective. Previous reports indicated that the temperature of cleaning solution could affect the type of microorganisms on milking equipment surfaces. The predominance of thermoduric over other microflora from the milking equipment may be related to the use of high temperature during cleaning of the equipment, whereas, the dominance of thermolabile species such as pseudomonas and coliforms, indicate the use of low (<42°C) cleaning temperature (1, 16).

In our study, CC controls corresponded with using either medium or high cleaning temperature, whereas CC cases were related to the use of low cleaning temperature. However, low LPC was associated with high water temperature which

indicates that the dominance of specific type of micro-organisms from milking equipment is influenced by additional factors such as chemical cleaning.

The majority of cleaning chemicals related factors were associated with LPC and CC. High water hardness score was associated with elevated LPC and CC, whereas, high alkalinity of the detergent wash was a risk for all bacterial counts. Very hard water or use of highly alkaline cleaners enhances the development of milk-stone. Additionally, too high alkalinity may also lead to corrosion of surfaces and increase deterioration of rubber parts and gaskets which provide conditions for bacterial adherence and formation of biofilms (19). Microorganisms such as micrococci, enterococci, coliforms, aerobic spore-formers, certain lactobacilli and other Gram-negative bacteria become embedded in the biofilm, multiply in it, and are protected from the effects of detergent and disinfectant solutions (20). Based on the composition of the microflora embedded in the biofilm, it could be associated with an elevated LPC or CC or PIC or TAC.

The associations between LPC, CC, and the majority of equipment hygiene related factors support previous reports by Thomas et al. (21) and Reinemann et al. (18) who indicated that improperly cleansed dairy equipment and bacterial incubation on milk contact surfaces are the main source of thermoduric and Gram-negative rods. These results also support the conclusion by Villar et al. (22) that thermoduric and coliform counts characterize the hygienic condition of dairy equipment.

Less frequent evaluation of water source was a risk for high counts in all quality parameters. An untreated water supply could be a source of contamination with coliforms, *pseudomonas* spp., and other Gram-negative bacteria (2), which could incubate on milking equipment and elevate CC and PIC. Furthermore, water hardness

minerals can react with cleaning agents and reduce their cleaning efficiency (18). Regular checking of water supply could also indicate a positive attitude of the farmers towards hygiene in general.

A high bulk tank outlet bioluminescence score was associated with elevated TAC, PIC and CC. The outlet valve is considered one of the major sources for contamination of raw milk stored in a bulk tank. This valve is difficult to clean and may allow accumulation of milk residues which provide good environment for bacterial growth (2).

Finally, although MCA does not assess the statistical significance of the relationship between independent variables and the outcome, it summarizes the complex relationships that exist among these variables. Our MCA results showed that preventive categories were tightly clustered around controls, whereas risk categories were more diffuse around cases (less strongly associated with cases). These results suggest that, control herds were doing most practices correctly. The results also imply that case herds did not necessarily do all practices wrong, however, failing to follow a comprehensive control system may cause the herd to be a case.

5.6 Conclusions

This study highlights the importance of different bacterial counts (TAC, PIC, LPC, and CC) as indicators of on-farm hygienic conditions during milk production. The TAC and PIC were mainly associated with environmental and cow hygiene, with poor hygiene scores being associated with elevated bacterial counts. The LPC and CC were mainly related to equipment hygiene with high temperature of the wash solution being

protective, whereas high water hardness score and high alkalinity of the detergent wash being risk factors.

5.7 Acknowledgements

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Table 5.1 Unconditional associations ($P < 0.15$) between environment and cow hygiene related factors and each of total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC), and coliform (CC) counts.

Parameter[1]	TAC	PIC	LPC	CC
Milking cow stall hygiene score	3.98[2***]	4.00[**]		
Dry cow stall hygiene score	7.42[***]	4.72[***]		
Cow hygiene score[3]	7.00[***]			
Milking cow udder hygiene score	7.71[***]			
Milking cow leg hygiene score	4.73[***]			3.22[**]
Milking cow flank hygiene score	3.24[**]			
Dry cow udder hygiene score	4.00[*]			
Teat cleanliness score	4.60[***]	5.52[**]		3.21[**]
Udder hair clipping	0.26[**]			
Teat wash	7.62[***]	3.77[**]		
Pre-dip	0.26[**]	0.31[**]		

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count, and CC = coliform count.
[2]Odds ratio.
[3] Average of udder, leg, and flank scores.
[***]$P < 0.01$ [**]$P < 0.05$ [*]$P < 0.15$

Table 5.2 Unconditional associations ($P < 0.15$) between equipment hygiene related factors and each of total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC), and coliform (CC) counts.

Parameter[1]	TAC	PIC	LPC	CC
Temperature (Celsius)				
Pre- wash fill temperature				0.93[2***]
Pre- wash drain temperature			0.92**	0.89***
Detergent wash temperature score[3]			0.89***	0.89***
Bulk tank alkaline wash fill temperature		0.93**	0.95*	0.90***
Bulk tank alkaline wash drain temperature			0.89***	0.93**
Pipeline alkaline wash start temperature			0.91**	0.92**
Pipeline alkaline wash end temperature	0.92*		0.86**	
Chemistry				
Bulk tank alkaline wash alkalinity (ppm)			1.001**	
Bulk tank alkaline wash chlorine >100 ppm				0.24**
Bulk tank alkaline wash pH			4.3*	
Bulk tank acid rinse (yes vs. no)				0.22*
Water hardness score (gpg[4])			1.33*	1.25**
Pipeline alkaline wash alkalinity (ppm)	1.003**	1.002**		1.002*
Pipeline alkaline wash chlorine >100 ppm			0.23**	
Pipeline alkaline wash pH	2.43*			3.23*
Other equipment				
Slug score	0.84*		0.79*	
Bulk tank automatic cleaning				0.12***
Milk house water bacterial check: yearly vs. longer	2.85*	2.85*	8.75**	3.52*
Milk house water hardness check: yearly vs. longer		2.42*	7.00**	3.24*
Bulk tank outlet bioluminescence score >0	2.62*	2.43*		3.91*
Water softener (yes vs. no)	0.32*	0.08***		

[1]TAC = total aerobic count, PIC = preliminary incubation count, LPC = laboratory pasteurization count, and CC = coliform count.
[2]Odds ratio.
[3] Average of alkaline wash start and end temperatures of bulk tank and pipeline.
[4]gpg = grain per gallon.
***$P < 0.01$ **$P < 0.05$ *$P < 0.15$

Table 5.3 Categories of the continuous predictors that were used for correspondence analysis.

Variable	Range	Percent	Merging categories[1]
Cow hygiene score(udder, leg, and flank)	1 - 3.25		
Low <1.3		25%	Low and medium (TAC)[2]
Medium 1.31-1.99		50%	
High ≥2		25%	
Teat cleanliness	1 - 3.12		
Low <1.1		25%	Low and medium (TAC)
Medium 1.11- 1.69		50%	
High ≥1.7		25%	
Pipeline alkaline wash alkalinity (ppm)[3]	0 - 1500		
Low <250		9%	Low and medium (TAC)
Medium 250-500		53%	
High ≥500		38%	
Bulk tank alkaline wash alkalinity (ppm)[3]	0 - 4000		
Low <400		32%	
Medium 400-800		43%	
High ≥800		25%	
Bulk tank alkaline wash fill temperature °C[3]	8.3 – 80		
Adequate ≥71		25%	
Inadequate <71		75%	
Detergent wash temperature score °C[4]	23.5-70.5		
Low < 50		25%	
Medium 50-57		50%	
High >57		25%	

[1]Low and medium categories were merged if they were originally located close to each other in correspondence analysis graph.
[2]TAC = total aerobic count.
[3]Thresholds were selected according to IDF Bulletin (Reinemann et al., 2003).
[4]Average of alkaline wash start and end temperatures of bulk tank and pipeline.

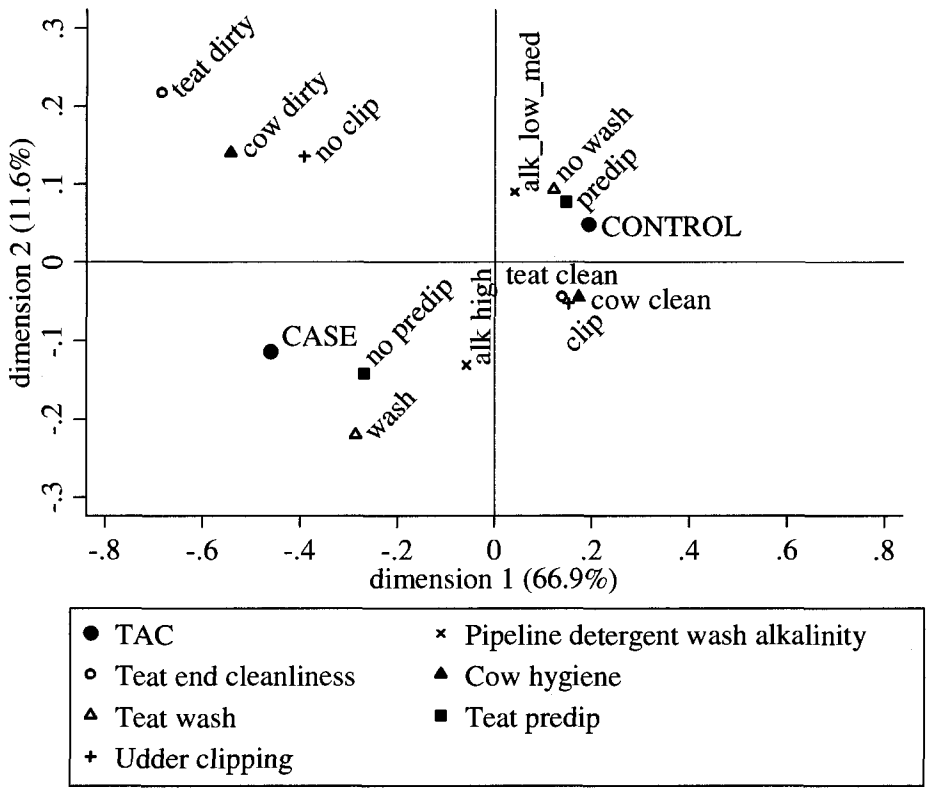

Figure 5.1 Multiple correspondence analysis of risk factors for high total aerobic count (TAC) in bulk tank raw milk in Prince Edward Island dairy herds.

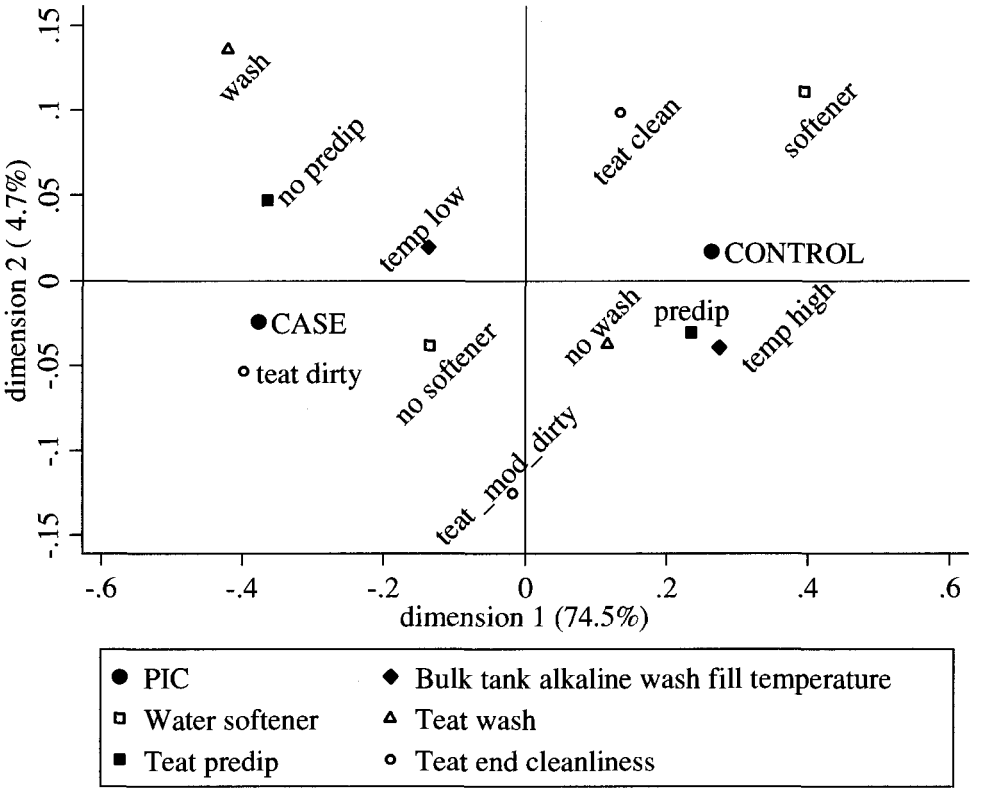

Figure 5.2 Multiple correspondence analysis of risk factors for high preliminary incubation count (PIC) in bulk tank raw milk in Prince Edward Island dairy herds.

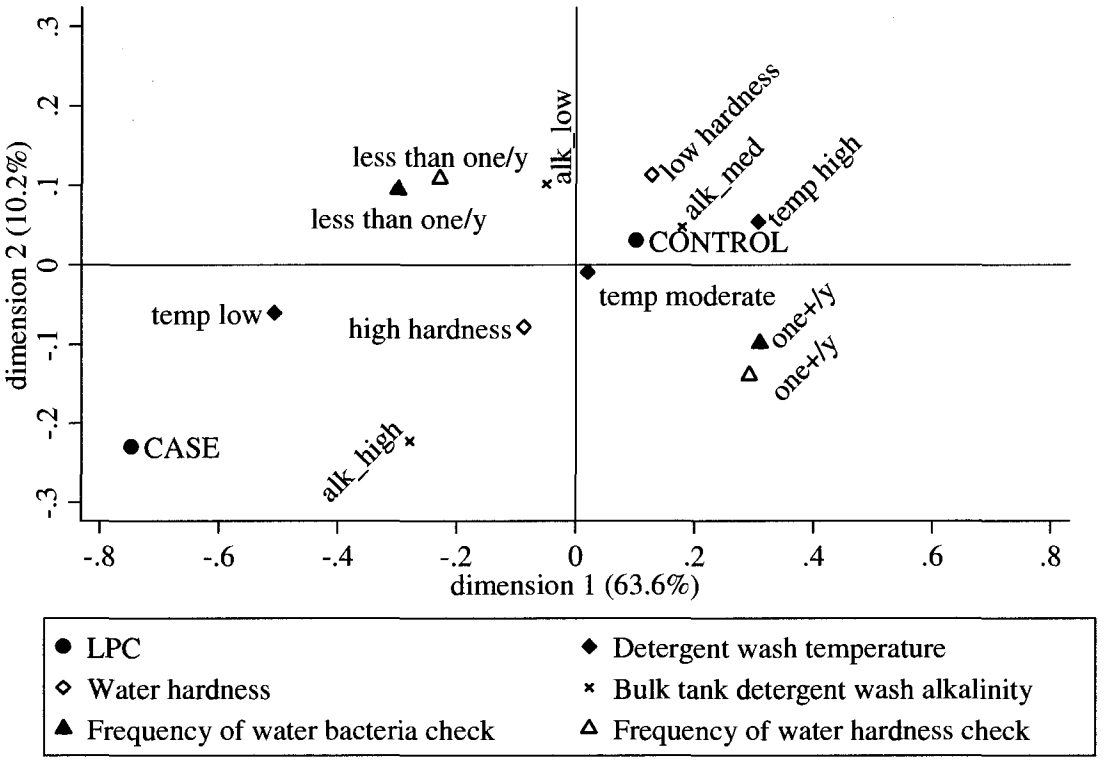

Figure 5.3 Multiple correspondence analysis of risk factors for high laboratory pasteurization count (LPC) in bulk tank raw milk in Prince Edward Island dairy herds.

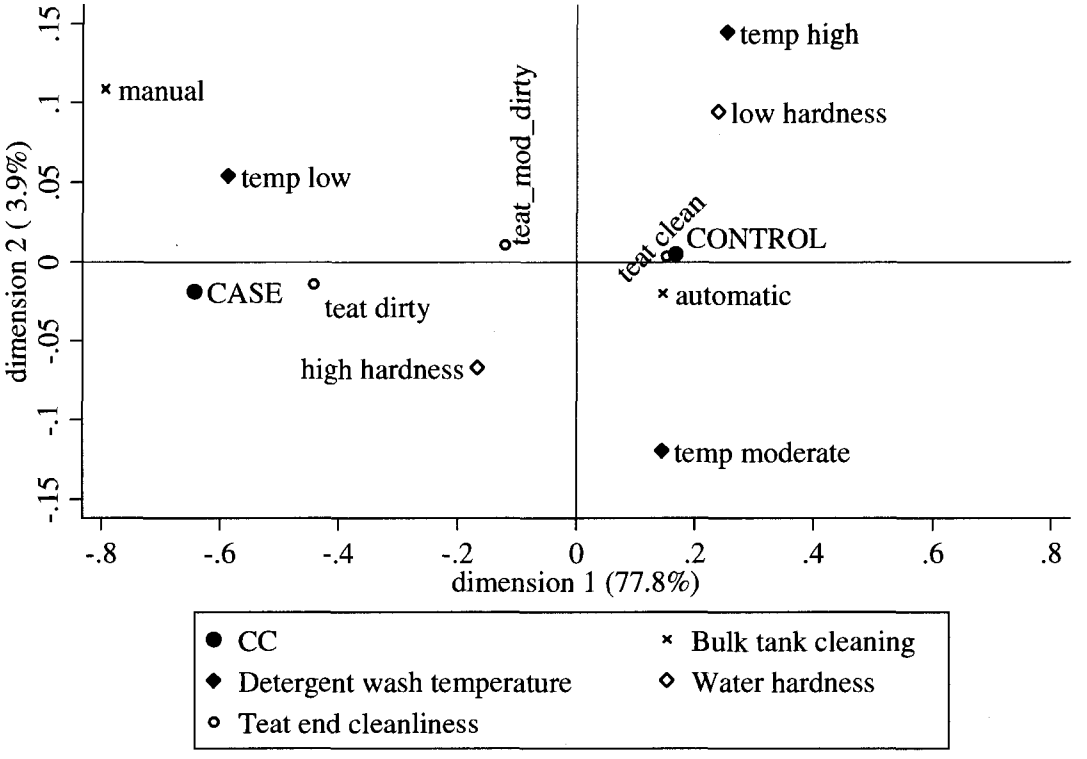

Figure 5.4 Multiple correspondence analysis of risk factors for high coliform count (CC) in bulk tank raw milk in Prince Edward Island dairy herds.

CHAPTER 6. IDENTIFICATION AND CHARACTERIZATION OF LIPOLYTIC AND PROTEOLYTIC BACTERIA IN PASTEURIZED MILK

6.1 Abstract

The objective of this study was to identify bacteria which survive laboratory pasteurization and characterize their lipolytic and/or proteolytic activity. Bulk tank milk from 100 dairy farms was subjected to laboratory pasteurization (62.8 °C for 30 min). Bacteria that survived pasteurization were sub-cultured onto spirit blue agar and skim milk agar and stored at 12.8 °C for 14 days to determine their lipolytic and proteolytic activity, respectively. Isolates that showed lipolysis and/or proteolysis were further characterized using Gram stain, morphology, and API® - based identification.

The predominant microorganisms were Gram-positive rods (83%) followed by Gram-positive cocci (17%). *Bacillus* spp. were common among Gram-positive rods. *Bacillus pumilus* accounted for 47% of the total isolates. Among the 8 staphylococci isolates, two isolates were *Staphylococcus aureus.* In our study, the absence of Gram-negative psychrotrophs is likely due to the limited risk of sample recontamination compared to commercial pasteurization. From the results of this study it can be concluded that: 1) Gram-positive bacteria dominated spoilage microorganisms in the absence of post-pasteurization contamination by Gram-negative bacteria, 2) under poor refrigeration conditions for two weeks, *Bacillus* spp. dominated among spoilage bacteria, and 3) most of the isolates had proteolytic or lipolytic activity or both which indicate their potential of causing spoilage of pasteurized milk.

6.2 Introduction

Microbial spoilage is considered the major detrimental factor for the shelf-life of pasteurized milk. Spoilage bacteria may be attributed to: 1) post-pasteurization contamination by Gram-negative psychrotrophic bacteria, which generally do not survive pasteurization, 2) the presence, in raw milk, of thermoduric and spore-forming bacteria, which survive the pasteurization process (1), 3) production of proteolytic and lipolytic enzymes by heat labile bacteria before pasteurization, and 4) enzyme production by thermoduric bacteria during refrigerated storage of milk after pasteurization. These enzymes can resist pasteurization and even ultra high temperature (UHT) processing (2).

Chemical components of milk can be degraded by bacterial metabolism and the activity of extracellular enzymes secreted by bacteria. Proteases hydrolyze casein and form bitter-tasting peptides; cause curdling and clotting of the milk; result in production of ammonia and hydrogen sulfide; and cause gelation of the milk. Lipases break down triglycerides, create short chain fatty acids that give milk a rancid smell and taste. Phospholipases hydrolyze phospholipids present in fat globule membranes making interior lipids more susceptible to the action of lipases (2).

Research into the bacterial spoilage of pasteurized fluid milk has focused predominantly on post-pasteurization contamination by Gram-negative psychrotrophs, such as *Pseudomonas* spp. However, improved processing conditions for milk and other dairy products have decreased the relative importance of these non-heat-resistant contaminants and revealed the presence of Gram-positive psychrotrophs as the next hurdle to extending the shelf-life of pasteurized fluid milk (3). It has been estimated that 25% of all shelf-life problems associated with conventionally pasteurized milk and cream

products in the United States may be linked to this class of thermoduric bacteria, with a large number of the contaminants being psychrotrophic *Bacillus* spp. (4). Therefore the objective of this study was to identify and characterize lipolytic and proteolytic bacteria, which survive the pasteurization process.

6.3 Materials and Methods

6.3.1 Microbiological analyses

This study was a part of larger study designed to evaluate the association between bacteriological quality of bulk tank milk and on-farm management practices in Prince Edward Island dairy herds. In the larger study, all herds (n=235) in the province were tested biweekly for bacterial quality, including laboratory pasteurization count (LPC). For the current study, bulk tank milk samples from all farms were collected during July 2006. Two aliquots were prepared from each sample. The first aliquot was screened using LPC methods on Petrifilm (3M Canada, London, Ont.) at the Atlantic Veterinary College. Raw milk samples from the second aliquot that corresponded to LPC > 25 cfu/ml (n=50) and 50 randomly selected samples from the low LPC group (≤25 cfu/ml) were frozen at -20 °C for further evaluation. The 25 cfu/ml cut point represents the 75th percentile of LPC for the milk samples tested over the year preceding this study.

At Pennsylvania State University, raw milk samples were thawed and subjected to LPC by heating raw milk samples to 62.8 °C for 30 min, then immediately cooled to below 10 °C and plated onto plate count agar (PCA) using the spiral plate count method (Autoplate 4000, Spiral Biotech, MA, USA). Additionally, milk samples were also plated on spirit blue agar (SBA) and skim milk agar (SMA) to determine their lipolytic and proteolytic activity, respectively. All culture methods were done according to the

Standard Methods for Examination of Dairy Products (5). Plates for LPC determination were incubated at 32°C for 48 h. The SBA and SMA plates were incubated at 12.8 °C for 14 days to approximate poor refrigeration conditions. The presence of clear or dark blue zones around the colonies on SBA was indicative of lipolysis, whereas the presence of clear zones around the colonies on SMA was evidence of proteolysis (5).

Colonies with different morphologies from SBA and SMA plates that showed enzymatic activity were sub-cultured onto blood agar and plates were incubated at 37 °C for 24 h. Colony morphology and hemolytic patterns on blood agar were observed and isolates were examined further by means of Gram staining, catalase, and oxidase testing. Identification of the isolates (n=75) to the genus and species level was carried out using API 50 CHB and API STAPH (bioMerieux, Durham NC).

6.3.2 Statistical analyses

Colony count data for LPC were strongly skewed to the right and could not be normalized by transformation. Therefore the difference in the median LPC counts between milk with enzymatic and milk without enzymatic activity was tested using the non-parametric Mann-Whitney test. The frequency of samples with and without enzymatic activity among the low and high LPC groups was compared using the Chi squared test.

6.4 Results

The LPC for milk with enzymatic activity ranged from 20-5,000 cfu/ml with a median of 36 cfu/ml, whereas for milk without enzymatic activity, LPC ranged from 0-1,900 cfu/ml with a median of 16 cfu/ml. The difference in the median count between the

two groups was significant ($P < 0.01$). Additionally, 49% of the high LPC (> 25 cfu/ml) group had enzymatic activity compared to 24% in low (≤ 25) LPC group ($P < 0.01$).

At an incubation temperature of 12.8 °C for 14 days, 75 isolates with enzymatic activity were obtained from 36 different farms (12 from the low LPC group, and 24 from the high LPC group). Table 6.1 illustrates the species profile and proteolytic and lipolytic activity of these organisms. The predominant (among those showing enzymatic activity) microorganisms were Gram-positive rods (83%) and Gram-positive cocci (17%). *Bacillus* spp. was common among Gram-positive rods. *Bacillus pumilus* was predominant among all isolates and accounted for 47% of the total isolates. Among the isolates of staphylococci (n=8), two were identified as *Staphylococcus aureus*. No Gram-negative organisms were isolated from laboratory pasteurized milk samples.

6.5 Discussion

The keeping quality of pasteurized milk is correlated with the thermoduric count of raw milk from which it was manufactured (4). In this study, higher thermoduric counts were observed for milk with proteolytic and or lipolytic activity than in milk without enzymatic activity. These results support previous findings (4) and indicate that enzyme production could be influenced by bacterial level in milk.

Our results show that *Bacillus* spp. was the predominant spp. accounting for 85% of Gram-positive rods. The predominance of Gram-positive rods over other microbial communities agree with previous work by Fromm and Boor (1) who reported that Gram-positive rods constituted 87% of processed milk microbial isolates. Griffiths and Phillips (6) isolated psychrotrophic *Bacillus* spp. from 69% of pasteurized milk samples, whereas

Fromm and Boor (1), found that the predominant spp. were *Paenibacillus* (39%) and *Bacillus* spp. (32%). These results support previous reports by Griffiths and Phillips (6) and Ralyea et al. (3) who indicated that more effective control of post-pasteurization contamination has resulted in the emergence of Gram-positive psychrotrophic bacteria as an important source of spoilage of high temperature short time (HTST) pasteurized fluid milk. In our study, the predominance of *Bacillus* spp. may be related to the time period over which the sampling was performed (July 2006), because higher incidence of *Bacillus* spp. has been observed during summer (7). Another factor that may be related to the predominance of *Bacillus* spp. was the incubation temperature used in this study (12.8 ^{o}C), because thermoduric spore-formers dominate spoilage bacteria at temperature >10 ^{o}C (8).

Our finding that *Bacillus pumilus* was the predominant *Bacillus* spp. is in contrast with the previous findings in which either *B. cereus* and *B. licheniformis (1),* or *B. cereus* and *B. mycoides* (6), or *B. mycoides* (9) were the predominant *Bacillus* spp. isolated from pasteurized milk. However, *B. pumilus* was also isolated from pasteurized milk in the previous studies. The 3 studies used API system for identification of *Bacillus* spp. except study (1) which used combination of API strips and 16s rDNA sequencing. In this study, the predominance of *B. pumilus* over *B. cereus* may be related to the incubation temperature used in this study (12.8 °C). Garcia et al. (10) reported that psychrotrophic *B. cereus* grew better at 6.5 °C than other psychrotrophic *Bacillus* spp.

Collectively, *Bacillus* spp. represent a specific concern for spoilage of milk and milk products due to their ability to survive pasteurization temperature and grow at refrigerated storage after pasteurization. Chen et al. (11) indicated that 86 % of

thermoduric psychrotrophic bacteria isolated from raw milk were *Bacillus* spp., which also produces heat-stable lipolytic and proteolytic enzymes resulting in spoilage problems.

Gram-positive cocci comprised 17% of the total isolates, with *Staphylococcus* spp. accounting for 13%. More interestingly, two of the isolates were identified as *S. aureus*. This finding contradicts the widely accepted belief that *S. aureus* does not survive either batch or HTST pasteurization process. However, *S. aureus* had been isolated before from milk after laboratory pasteurization at 62.8 °C for 30 min (12). A recent study showed that *S. aureus* is more thermotolerant than *Listeria monocytogenes* and can survive cooking conditions of 70 °C for 2 min (13). Additionally, Rodriguez and Yousef (14) indicated that exposure of microorganisms to a type of stress may lead to cross-protection against another type of stress. Furthermore, Shebuski et al. (15) showed that thermotolerance of *S. aureus* dramatically increased in media with low water activity. In our study, milk samples were frozen at -20 °C before the pasteurization process, therefore reduced water activity due to freezing may have increased thermal resistance of *S. aureus* and hence its survival of laboratory pasteurization. Although, previous literature provides some evidence that *S. aureus* may survive pasteurization conditions, the possibility of skin contamination from laboratory persons cannot be ruled out. The fact that no Gram negative psychrotrophs were isolated in the post-pasteurized samples indicates that laboratory contamination containment procedures were effective.

In our study, the absence of Gram-negative psychrotrophs is likely due to the limited risk of sample recontamination after laboratory pasteurization compared to commercial pasteurization. Sørhaug and Stepaniak (2) stated that post-pasteurization

contamination by *Pseudomonas* spp. is the most detrimental factor for the keeping quality of HTST pasteurized milk. Our results indicate that proper handling of milk after pasteurization can eliminate Gram-negative spoilage problems.

The proteolytic and/or lipolytic activity showed by most of the isolates suggests that the identified species have the potential of causing spoilage of pasteurized fluid milk. Proteolytic activity has been associated with gelation of UHT milk, sweet curdling of milk, bitter and unclean flavors in cheese, decrease in cheese yield, and textural defects in cultured dairy products, whereas lipolytic activity can lead to rancid and fruity off-flavors in milk products (4).

In addition to the enzymatic activity and effect on milk quality, some of the identified bacteria are of public health importance such as *B. cereus* and *S. aureus*. Moreover, food poisoning associated with *B. licheniformis* and *B. pumilus* has also been documented (16). In fact, Griffiths (17) has detected a diarrhegenic toxin associated with a psychrotrophic strain of *B. pumilus* isolated from milk.

6.6 Conclusions

With the control of post-pasteurization contamination by Gram-negative bacteria, the presence of thermoduric bacteria (particularly *Bacillus* spp.) and the activity of heat stable proteolytic and lipolytic enzymes produced by some psychrotrophs in milk will be the limiting factor for the shelf-life of fluid milk and dairy products. The isolation of *S. aureus* from pasteurized milk in this study requires further investigations to rule out post-pasteurization contamination from laboratory personnel.

6.7 Acknowledgement

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Table 6.1 Species profile and proteolytic and lipolytic activity of bacterial isolates that survived laboratory pasteurization.

Species	No. of farms[1]	No. of isolates	% isolates	Proteolysis	Lipolysis
Bacillus spp.					
B. pumilus	24	35	46.7	11	24
B. cereus	6	6	8.0	4	2
B. circulans	3	3	4.0	0	3
B. licheniformis	2	2	2.7	1	1
B. stearothermophilus	2	2	2.7	0	2
B. mycoides	1	1	1.3	0	1
Brevibacillus brevis	2	3	4.0	3	0
Staphylococcus spp.					
S. aureus	1	2	2.7	0	2
S. chromogenes	1	2	2.7	0	2
S. epidermidis	1	1	1.3	0	1
S. xylosus	1	1	1.3	1	0
S. sciuri	2	2	2.7	2	0
Lactococcus lactis	2	3	4.0	0	3
Micrococcus spp	1	1	1.3	1	0
Unidentifiable	7	11	14.7	3	8
Total		75	100.0	26	49

[1] The total number of unique farms is 36. Some farms had multiple isolates.

CHAPTER 7. MASTITIS PATHOGENS ASSOCIATED WITH ELEVATED BACTERIAL COUNT IN BULK TANK MILK OF PRINCE EDWARD ISLAND DAIRY HERDS

7.1. Abstract

The aim of this study was to investigate the role of bacteria that may be associated with mastitis as a source of elevated total bacterial counts in bulk tank milk of Prince Edward Island dairy herds. Bulk tank milk samples from all 235 dairy herds were collected over a 6 week period and scanned for total bacterial count using the Bactoscan method. Samples that had a Bactoscan count >8,000 bacteria/ml (n=128 from 90 farms) were further analyzed for total aerobic count on Petrifilm, and for streptococcus, staphylococcus, and Gram-negative bacterial counts using specific media.

Counts >1,000 cfu/ml for streptococci and staphylococci were present in 35% and 41% of the 128 bulk tank milk samples, respectively, while 30% of Gram-negative bacterial counts were >500 cfu/ml. In 17 (19%) out of 89 milk samples that had total aerobic count >10,000 cfu/ml, bacteria that may be associated with mastitis contributed a proportion >0.25 to the total aerobic count. *Streptococcus uberis, Escherichia coli*, and coagulase-negative staphylococci were the main contributors. In 4 instances, counts >80,000 cfu/ml had been contributed by *S. uberis and E. coli*. In conclusion, while the majority of high bacterial counts were not associated with organisms that may be associated with mastitis, these pathogens can be present in very high numbers in bulk tank milk and can contribute to high total bacterial count. Further knowledge of individual cow culture will be required to determine whether these pathogens were from intramammary infection or if environmental source lead to contamination of the surface of the udder and teats.

7.2 Introduction

Bacteriological examination of bulk tank milk (BTM) has been shown to be a useful tool in monitoring and investigating current and potential milk quality and mastitis problems in dairy farms (1, 2). Bacterial quality of BTM can be measured by several tests including estimation of total aerobic count (TAC). The TAC is a recognized alternative to the standard plate count and gives an indication of the total number of aerobic bacteria in raw milk samples and is an important parameter in regulatory and quality incentive programs in many parts of the world. The TAC reflects general hygienic conditions during milk production (3).

A high bacterial count in BTM may be associated with bacterial contamination from intramammary infection, from the external surface of the udder and teats, and from the surface of improperly cleaned milking equipment (4). Specific risk factors associated with high TAC include dirty udders and teats, and washing the teats with water for premilking udder preparation, whereas protective factors include udder hair clipping and using teat predip (5).

Intramammary infections, which can be clinical or subclinical, reduce milk yield, alter milk composition, and elevate somatic cell and bacterial counts (6-8). Although inclusion of milk from cows with subclinical mastitis has been reported to contribute $<10^4$ cfu/ml to the BTM, accidental inclusion of milk from cows with clinical mastitis may elevate bacterial counts in BTM to 10^5 cfu/ml (7).

Jeffrey and Wilson (9) reported that about 44% of Scottish milk samples with TAC >45,000 cfu/ml were dominated by bacteria that may be associated with mastitis, with *Str. uberis* dominating in 18% of the samples. In a Danish study, mastitis associated

pathogens were found in 48% of milk with total bacterial count >30,000 cfu/ml and were the main cause of elevated microbial count in 8% of the samples (10). In New York, Zadoks et al (11) revealed that mastitis causing streptococci were important contributors to total bacterial count in BTM and may be present at levels >100,000 cfu/ml.

In Prince Edward Island (PEI), no studies have been done to investigate the role of mastitis associated pathogens in elevated total bacterial count in BTM. The initial objective of this chapter was to speciate BTM, which have high TAC and normal preliminary incubation count (PIC) for mastitis associated pathogens, and to compare microbial profile of this group to another group, with both high TAC and PIC. However, because all of the PIC samples were too high, we could not proceed with the initial objective. Therefore the aim of this study was to speciate BTM, with elevated total bacterial counts for bacteria that may be associated with mastitis in PEI dairy herds.

7.3. Materials and Methods

7.3.1. Milk samples

Bulk tank milk samples were collected from all PEI dairy herds (n=235) every other week over a six week period (June 12th to July 17th, 2007). Samples were collected in 30 mL sterile screw cap tubes (Starplex Scientific Inc., Etobicoke, Ont.) by trained milk haulers and held on ice until arrival at the laboratory. All samples were screened for total bacterial count at the PEI milk quality laboratory, using Bactoscan 50 (FOSS Electric, Hillrød Denmark). Samples that had a Bactoscan count >8,000 bacteria /ml were retained for further evaluations at the Atlantic Veterinary College.

7.3.2. Microbiological examination

Milk samples were mixed thoroughly by vortex and 2 subsets from each sample were prepared: 1) whole milk samples, 50 μL was plated onto each of the following media using a spiroplater (Autoplate 4000, Spiral Biotech, MA, USA): Edward's modified agar supplemented with colistin sulfate and oxolinic acid (EMCO) for enumeration of streptococci, Baird Parker agar (BP) for estimation of staphylococci, and MacConkey agar (MA) for evaluation of Gram-negative bacteria, and 2) diluted milk samples, 0.01 and 0.001 dilutions were used for enumeration of TAC on Petrifilm aerobic count plates. Plates for enumeration of TAC on Petrifilm were incubated at 32 °C for 48 h. The other plates (EMCO, BP, and MA) were incubated at 37 °C for 48 h.

Petrifilm plates were read using an automated counter (3M Petrifilm Plate Reader, 3M Canada, London Ontario), the other plates (EMCO, BP, and MA) were read using a spiral plate grid (Spiral Biotech, MA, USA) according to recommendations of the manufacturer.

Colonies from EMCO, BP, and MA plates were classified morphologically and 2 colonies from the primary (most frequent) and secondary (second most frequent) isolates were subcultured onto blood agar. These blood agar plates were incubated at 37 °C for 48 h and colony morphology and hemolytic patterns were observed. Isolates were examined further by means of Gram-staining and catalase and oxidase testing. Gram-positive, catalase-negative, CAMP-positive, and Esculin-negative cocci were considered *Streptococcus agalactiae*. Other streptococci were identified based on the results from CAMP, Hippurate, Inulin, and Esculin tests. *Staphylococcus aureus* was identified as Gram-positive, catalase-positive, and coagulase positive, with α- and β-hemolysis on

blood agar. Coagulase-negative staphylococci (CNS) were grouped as such. Gram-negative bacteria were identified by Gram-stain, lactose fermentation and indole, and oxidase testing.

7.3.3. Descriptive statistics

Data were collected on spreadsheets and merged into a single database using Stata version 10 (Stata Corp, College Station TX). Summary statistics and frequency distributions for TAC, streptococci, staphylococci, and Gram-negative bacterial counts were computed on raw data. Additionally, streptococci, staphylococci, and Gram-negative bacterial counts were categorized into low, medium, and high according to guidelines recommended by Jayarao et al. (2) and the percentage of each category was calculated. The contribution of each specific count to the TAC was determined by dividing each count by the TAC using raw data. Finally, the contribution of specific organisms to high TAC (>10,000 cfu/ml) was calculated for samples in which organisms that may be associated with mastitis contributed a proportion ≥0.25 to the elevated TAC.

7.4. Results

7.4.1. Bacterial count

Over the 6 weeks study period, from approximately 705 BTM samples tested, a total of 128 samples from 90 unique farms had a Bactoscan count >8,000 bacteria/ml. Therefore, they were eligible for further evaluation.

Table 7.1 shows summary statistics for different bacterial counts of the 128 BTM samples. The TAC ranged from 1,100-850,000 cfu/ml, with a median count of 17,000 cfu/ml. The median streptococci, staphylococci, and Gram-negative bacterial counts were

560, 760, and 240 cfu/ml, respectively. Using the thresholds for high counts recommended by Jayarao et al (2), 35% of the samples with a Bactoscan count > 8,000 bacteria/ml had a streptococci count >1,000 cfu/ml, 41% had a staphylococci count > 1,000, and 30% had a Gram-negative count >500 cfu/ml (Table 7.2).

7.4.2. Frequency of isolation of specific bacteria

Table 7.3 shows the frequency of streptococci, staphylococci, and Gram-negative bacteria on specific media. On the EMCO plates, isolates identified as *Streptococcus* spp. were the most common and were the primary isolate in 80 (62.5%) of the 128 EMCO plates. With regard to Baird Parker plates, most of the isolates were identified as CNS. The CNS were the primary isolate in 100 (78%) of the 128 BP plates. The growth of Gram-negative bacteria on MA plates showed that *Escherichia coli* was the most common isolate among Gram-negative bacteria and was the primary isolate in 50 (39%) of 128 MA plates.

7.4.3. Contribution of bacterial species to total count

Table 7.4 shows the percentiles of the proportions of the TAC that were contributed by each of streptococci, staphylococci, and Gram-negative bacterial counts. At a TAC level <10,000 cfu/ml, streptococci and staphylococci were the main proportional contributors to TAC (10% were ≥0.39 and 0.71, respectively). At a TAC level between 10,000 and 30,000 cfu/ml, streptococci and staphylococci were also the main contributor to TAC, but their proportional contribution to the TAC decreased (10% were ≥0.22 and 0.21, respectively). In general, Gram-negative bacteria contributed less to the TAC than streptococci and staphylococci. However, in a few samples with TAC >30,000 cfu/ml, Gram-negative bacteria made a significant proportional contribution

(10% were ≥0.26), but streptococci and staphylococci did not (10% were ≥0.12 and 0.06, respectively).

Table 7.5 shows 17 samples (19%) in which mastitis associated pathogens contributed a proportion >0.25, when the TAC was >10,000 cfu/ml. Streptococci were the main contributors in 8 samples. *Streptococcus uberis* was the predominant isolate in 6 of those 8 samples and reached counts >92,000 cfu/ml (the maximum count possible on a plate). Staphylococci were the main contributors in 4 samples, with the highest count being 23,000 cfu/ml. Gram-negative bacteria were the main contributors in 5 samples with *E.coli*, *Enterobacter* spp., and *Pseudomonas* spp. being the dominant isolates from these samples, of which two had count >92,000 cfu/ml.

7.5. Discussion

The objective of this study was to determine differential contribution of various bacteria to elevated TAC in BTM, with a focus on organisms that may be associated with mastitis. In this study, milk samples were selected based on Bactoscan count >8,000 bacteria/ml. Approximately, 20% of the samples estimated by the Petrifilm method had a TAC <8,000 cfu/ml. The reason for these variations is that Bactoscan counts individual bacterial cell/ml, whereas the Petrifilm culture method counts colony forming unit/ml. Additionally, Bactoscan counts all bacteria irrespective of their cultural requirements, whereas Petrifilm counts organisms that can grow on basic media at selected temperatures (1).

For the 128 high Bactoscan counts, 35% of the total streptococci counts exceeded the limit for a high count (>1,000 cfu/ml) that has been suggested by Jayarao et al. (2).

The great majority of streptococci were environmental streptococci. These organisms are widely distributed in the cow's environment, including teat ends, teat skin, bedding, and feces. An increase in the number of these organisms in BTM may indicate problems related to environmental and milking hygiene in the herd (12) or may indicate a mastitis associated rise in TAC . *Streptococcus agalactiae* was isolated from 3 (2.3%) milk samples. This value is close to previous report by Olde Riekerink et al. (13) who reported a herd prevalence of 1.6% of *S. agalactiae* in Prince Edward Island. *Streptococcus agalactiae* is an obligate intramammary pathogen, therefore its presence in BTM is a positive indication of intramammary infection in a herd (14, 15). Bulk tank milk samples from *S. agalactiae* infected herds can be associated with elevated TAC due to shedding of large numbers of bacteria in milk (14, 15).

Forty one percent of staphylococci counts were >1,000 cfu/ml. In most of these samples, CNS were the primary staphylococci isolate. High counts of CNS in BTM could be caused by inadequate milking procedures, poor hygiene, teat skin irritation, and lack of teat dipping (16, 17) or from inside the udder.

Finally, while 23% of the 128 high Bactoscan samples had no growth of Gram-negative bacteria, 30% of the samples exceeded the threshold for high count (>500 cfu/ml). These huge variations in Gram-negative counts among herds indicate that hygienic conditions of these farms were very different.

In the majority (81%) of the 89 milk samples that had a high TAC (>10,000 cfu/ml), organisms that may be associated with mastitis contributed a proportion <0.25 to TAC. However, in 17 (19%) out of 89 samples, these pathogens had a significant contribution (≥0.25) to the TAC. *Streptococcus uberis* was the most common mastitis-

associated pathogen that contributed to elevated TAC. In 6 (6.6%) milk samples, *S. uberis* contributed a proportion >0.25 to the TAC. The primary source of this organism was not determined however, Hayes et al. (3) reported clinical and subclinical infection with *S. uberis* to be the cause of elevated TAC in BTM following isolation of this organism in the absence of other environmental contaminants such as *E. coli, Klebsiella* spp., and *Bacillus* spp. In another study, Zadoks et al. (11) showed that streptococci, mainly *S. uberis,* were important contributors to high TAC in BTM. Our results agree with the previous reports in that *S. uberis* was the most frequently isolated among mastitis-associated pathogens that had a significant contribution to an elevated TAC. However, in our study, data on individual cow cultures were not available, therefore the source of *S. uberis* could be from the inside or the outside of the udder.

Staphylococci contributed a proportion >0.25 to elevated TAC on 4 (4.5%) occasions. Coagulase-negative staphylococci were the main contributor to the TAC in 2 samples, and in the other 2 samples, both CNS and *S. aureus* contributed to the TAC. The primary source of *S. aureus* in a dairy herd is inside the udder of infected animals, therefore detection of this organism in BTM indicates presence of intramammary infection in the herd (18). *Staphylococcus aureus* is shed from infected udders intermittently and in low numbers, therefore mastitis caused by *S. aureus* in a herd will rarely elevate the TAC (19).

Coagulase-negative staphylococci are opportunistic pathogens and form part of the normal flora of the teat skin. Under favorable conditions, they can colonize the teat end or teat canal and subsequently produce mastitis (20). The source of CNS could be from intramammary infection or from the exterior of the udder and teats.

Gram-negative bacteria include coliforms such as *E. coli, Klebsiella* spp., and *Enterobacter* spp. and non-coliform such as *Pseudomonas* spp. Coliforms are ubiquitous in the farm environment and can be found in fecal and bedding material, and poorly cleaned milking equipment (7). Pseudomonas contamination can originate from an untreated water supply and poor hygiene on the farm (7). Among coliforms, *E. coli* has been shown to elevate TAC in BTM (3). In our study, Gram-negative bacteria were the main contributor to elevated TAC in 5 instances, with *E. coli* contributing a count >28,000 cfu/ml to an elevated TAC on 3 occasions. These high counts could be due to intramammary infection or environmental contamination. However, infected cows usually shed coliform organisms for only a short period of time, therefore the number of coliform bacteria in BTM is likely related to environmental contamination (21) or inadequate cleaning of the milking system (5).

In 4 of the samples in which mastitis-associated pathogens contributed >0.25 to TAC, the total proportion contributed by mastitis-associated pathogens was greater than one. This result seems counterintuitive because selective media typically isolate a subset of those organisms that would grow on a non-selective medium. The growth of microorganisms particularly streptococci on specific media could be enhanced due to inclusion of certain nutrient materials which are not available in non-selective media. Additionally, the presence of inhibitors in the specific media eliminates competition for nutrients from other microorganisms. Therefore, it is possible that microorganisms such as streptococci grew faster on the EMCO plates than on non-selective plates (3).

In conclusion, the results of this study indicate that in most of the cases, high TAC in BTM was not associated with pathogens that may be associated with mastitis.

However in some cases, mastitis-associated pathogens contributed a significant amount of bacteria to elevated TAC in BTM. Because many of these pathogens have an environmental reservoir, further knowledge of individual cow culture and prevalence of mastitis in herds will be required to determine whether the source of these organisms is intramammary infection or contamination from the exterior of the udder and teats.

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Table 7.1 Descriptive statistics of total aerobic, streptococci, staphylococci and Gram-negative bacteria counts from 128 bulk tank milk samples with Bactoscan count >8,000 bacteria/ml in Prince Edward Island.

Parameter	Min	Max	Percentiles		
			25%	50%	75%
TAC[1]	1100	850000	8500	17000	34500
Streptococci count[2]	0	>92000	260	560	1560
Staphylococci count[2]	0	23100	420	760	1740
Gram negative count[2]	0	>92000	20	240	690

[1] TAC=total aerobic count as measured by Petrifilm method using 0.01 and 0.001dilutions.
[2] Counts are measured by spiroplater using whole milk.

Table 7.2 Categories of streptococcus, staphylococcus, and Gram-negative bacteria of the 128 bulk tank milk samples with Bactoscan count >8,000 bacteria/ml in Prince Edward Island.

Species	Category[1]	Threshold (cfu/ml)[2]	Frequency	Percent
Streptococci	Low	<500	60	46.88
	Medium	500 - 1000	23	17.97
	High	>1000	45	35.16
Staphylococci	Low	<500	42	32.81
	Medium	500 - 1000	34	26.56
	High	>1000	52	40.63
Gram negative	Low	<250	66	51.56
	Medium	250 - 500	24	18.75
	High	>500	38	29.69

[1] Categories and threshold for each category based on guidelines by Jayarao et al (2).
[2] All counts were estimated by spiroplater using whole milk.

Table 7.3 Frequency of isolation of streptococcus, staphylococcus and Gram-negative bacteria and whether they were the primary or secondary isolate from 128 bulk tank milk samples with Bactoscan count >8,000 bacteria/ml in Prince Edward Island.

Species	Primary[1]	Secondary[2]
Streptococci on modified Edward's media (EMCO)		
Negative	3 (2.3)	NA[3]
Str. agalactiae	2 (1.6)	1 (2.6)
Str. dysgalactiae	5 (3.9)	2 (5.1)
Str. uberis	38 (29.7)	16 (41)
Streptococcus spp.	80 (62.5)	20 (51.3)
Total	128 (100)	39 (100)
Staphylococci on Baird Parker (BA)		
Negative	4 (3.2)	NA
S. aureus	24 (18.8)	44 (81.5)
CNS[4]	100 (78)	10 (18.5)
Total	128 (100)	54 (100)
Gram-negative bacteria on MacConkey agar (MA)		
Negative	29 (22.7)	NA
Pseudomonas spp.	10 (7.8)	17 (42.5)
Klebsiella spp.	19 (14.8)	6 (15)
Enterobacter spp.	20 (15.6)	6 (15)
Escherichia coli	50 (39.1)	11 (27.5)
Total	128 (100)	40 (100)

[1]Primary= most frequent colony type on specific plate. Percentage is shown in parentheses.
[2]Secondary= the second most frequent colony type on specific plate.
[3]NA=Not applicable.
[4]CNS= Coagulase negative staphylococci.

Table 7.4 Percentiles of the proportions of the total bacterial count that were contributed by streptococcus, staphylococcus, and Gram-negative bacteria. Data from 128 bulk tank milk samples with Bactoscan count >8,000 bacteria/ml in Prince Edward Island.

Bacterial species	10%	25%	50%	75%	90%
Observations with TAC <10,000 cfu/ml (n=39)	Proportion of the total bacteria load[1]				
Streptococci proportion	0.02	0.06	0.08	0.21	0.39
Staphylococci proportion	0.03	0.07	0.16	0.27	0.71
Gram negative proportion	0	0	0.01	0.04	0.10
Observations with TAC ≥10,000 & <30,000 cfu/ml (n=52)					
Streptococci proportion	0	0.02	0.04	0.11	0.22
Staphylococci proportion	0.01	0.02	0.05	0.09	0.21
Gram negative proportion	0	0	0.01	0.03	0.05
Observations with TAC ≥30,000 cfu/ml(n=37)					
Streptococci proportion	0	0	0.01	0.02	0.12
Staphylococci Proportion	0	0	0.01	0.02	0.06
Gram negative proportion	0	0	0.01	0.04	0.26

[1]Proportional contribution is < cell value for the row heading percentage of samples.

Table 7.5 Milk samples in which organisms that may be associated with mastitis contributed ≥ 0.25 of the total aerobic count (TAC) >10,000 cfu/ml (n=89). Data sorted by bacterial species and total proportion contributed by mastitis associated pathogens.

Dominant bacteria	TAC[1]	Strep[2]	Staph[3]	Gramneg[4]	Total prop[5]	Most contributing spp.
Streptococci	16000	7270	40	480	0.49	*S. uberis*
	23000	8820	2490	700	0.52	*Streptococcus.* spp
	14000	8730	560	0	0.66	*S. uberis*
	46000	34600	20	520	0.76	*S. uberis*
	21000	14800	1680	280	0.80	*Streptococcus.* spp
	20000	16200	1840	80	0.91	*S. uberis*
	76000	82400	200	0	1.08	*S. uberis*
	62000	92000	400	260	1.62	*S. uberis*
Staphylococci	14000	1680	5900	40	0.54	CNS
	17000	720	9190	600	0.62	CNS + *S. aureus*
	12000	1240	8360	440	0.84	CNS
	14000	340	23100	40	1.68	CNS + *S. aureus*
Gram negative	380000	720	2160	92000	0.25	Pseudomonas + Enterobacter
	70000	320	1240	28000	0.42	*E. coli*
	220000	200	300	92000	0.46	*E. coli*
	12000	260	0	6330	0.55	Enterobacter
	52000	1640	1560	36200	0.76	*E. coli*

[1] TAC=total aerobic count as measured by Petrifilm method using 0.01 and 0.001dilutions.
[2] Streptococci count as measured by spiroplater using whole milk.
[3] Staphylococci count as measured by spiroplater using whole milk.
[4] Gram-negative count as measured by spiroplater using whole milk.
[5] Total proportion contributed by mastitis associated pathogens.

CHAPTER 8. SUMMARY AND GENERAL DISCUSSION

8.1 Introduction

Although previous literature has emphasized that raw milk quality is essential for pasteurized milk quality, research data on specific on-farm risk factors for elevated bacterial counts in raw milk are very limited. Therefore, the overall objective of this thesis (Chapters 2-5) was to investigate the association between on-farm management practices and raw milk bacteriological quality as measured by total aerobic (TAC), preliminary incubation (PIC), laboratory pasteurization (LPC), and coliform counts (CC). During the course of the study, two non-risk factor studies were implemented to characterize thermoduric lipolytic or proteolytic bacteria (Chapter 6) and to speciate bulk tank milk (BTM) for mastitis pathogens (Chapter 7).

In Prince Edward Island, bacterial quality of milk used to be evaluated based on the SPC value alone, however, this test alone does not give a full assessment of the hygienic quality of raw milk. Therefore, in this project, in addition to the SPC or its alternative the TAC, 3 additional criteria were used for evaluation of raw milk bacterial quality including, PIC, LPC, and CC.

In order to address the overall objective, the following studies were performed:

8.2 Descriptive study on microbiological quality of bulk tank milk in PEI

In this study, BTM bacterial counts (TAC, PIC, LPC, CC, and SCC) were evaluated biweekly over a 2-year period. The objectives were to determine the current level (mean, median, and percentiles) for each of the milk quality parameters, to evaluate correlations among milk quality criteria, and to determine seasonal effect on milk quality parameters.

The great majority of samples tested for milk quality parameters in this study were below the regulatory limit of the province (TAC and SCC) or the acceptable limits suggested by the literature (PIC, LPC, and CC).

Bacterial counts reported in this study were lower than those reported in New York and Pennsylvania (1, 2) and were in the lower end of the range reported by Peeler et al. (3) in the study which involved 11 states. These variations in bacterial counts among different regions indicate that they can be influenced by different management practices.

In this work, the correlation between the various measures of quality was weak (<0.26) except for that between TAC and PIC, which was 0.57. This result is similar to the findings of others (1, 2, 4). In addition to indicating a lack of predictive ability among tests, this weak correlation suggests that each count gives different information in relation to management practices and sources of bacterial contamination. As a result, there is a need to use multiple tests to get a more complete assessment of the hygienic quality of raw milk.

With the exception of SCC, other milk quality parameters had moderate to high coefficients of variation (CV), which indicates that herd assessments should not rely on a single measurement.

The results regarding seasonal variation showed that there was no consistent seasonal pattern over the 2 year study period for TAC, PIC, and LPC, however, these quality parameters tended to have low median counts during winter. Coliform and SCC counts showed a similar pattern in both years, with the median counts being highest during the summer. The inconsistency of seasonal effects indicates that seasonal variations were mainly attributed to variations in management practices.

8.3 Risk factors for bacteriological quality of bulk tank milk

Associations between management practices and BTM bacterial quality were examined using a producer completed questionnaire (Chapter 3) and a case-control study (Chapter 4-5).

8.3.1 Survey on management practices

In this study, data for on-farm risk factors were collected via a mail-out survey which consisted of 4 main sections: general farm demographics and management, cow cleanliness and hygiene, milking procedures and mastitis control, and equipment maintenance and cleaning. One hundred and fifty three of the 235 herds in the province completed the survey for a response rate of 65%. The results of this study indicated that risk factors for elevated TAC and PIC were very similar, which may be partially explained by the moderate correlation (0.57) between the 2 tests shown in Chapter 2. The TAC and PIC were strongly associated with the proportion of cows soiled with manure, method of premilking udder preparation, and method of cleaning the bulk tank. The results related to premilking udder preparation showed that pre-dipping followed by drying the teats with single-use towel was associated with the lowest bacterial counts compared to other methods of teat preparation. These results highlight the importance of chemical sanitization and udder drying in premilking teat cleaning effectiveness, as has been reported by others (5, 6).

For LPC, having a water purification system decreased the risk, whereas having a plate cooler and inadequate acid wash frequency increased the risk. The negative effect of having a plate cooler was unexpected and may be associated with the difficulty of cleaning these devices. For CC, udder hair clipping and automated bulk tank washing

were protective, whereas being a large size herd and inadequate acid wash were risk factors.

The distribution of the variance from the mixed models used for the analysis of these data showed that most of the variations were attributed to within herd variations, which suggest that herd evaluation should rely on several measurements of each test and not on a single value. This conclusion was also suggested in Chapter 2 from the results of CV.

8.3.2 Case-control study (overall risk factors)

In this study, case and control herds were defined based on the last 6 results of the bacterial counts in BTM. Cases were herds which had multiple elevated counts for any of the parameters measured. Control herds had low bacterial count in all bacterial groups over the same period. A total of 69 herds (39 case and 30 control herds) were evaluated for on-farm hygiene and management practices. This evaluation included: observation of basic hygiene and farm management practices, complete wash analysis of the milking equipment, and scoring of environmental and cow hygiene. The results of this study demonstrated that poor teat end hygiene was associated with elevated bacterial counts in BTM, whereas, high water temperature of detergent wash and the use of a water softener were associated with low bacterial counts in bulk tank milk. Additionally, high alkalinity of alkaline detergent wash was also a risk factor. Increased detergent use by producers in response to high bacterial count problems may explain the apparent detrimental effect of higher alkalinity. Another explanation is that too much alkalinity may lead to corrosion of surfaces and increase deterioration of rubber parts and gaskets, which provide conditions for bacterial adherence and formation of biofilms. Other factors, with strong univariable

association, that did not remain in the final model included, udder hair clipping, udder hygiene score, water hardness score, checking water source for bacterial contamination and water hardness.

8.3.3 Case-control study (individual bacterial risk factors)

For examination of individual bacterial class (TAC, PIC, LPC and CC) cases versus controls, herds were defined on each specific bacterial class. Herds were classified as a case if they had multiple elevated counts in the last 6 analyses for that class. Control herds had 0/6 high counts for the specific bacterial class of interest. The results of this analysis showed that TAC and PIC were mainly associated with cow and stall hygiene, washing the teats with water and not using teat pre-dip, and having dirty teats after udder preparation. These results support the finding from the survey data in Chapter 3. The LPC and CC were related to equipment hygiene, with high counts being associated with low temperature of the cleaning solution, high water hardness score, and high alkalinity of alkaline detergent wash.

The results of the correspondence analysis showed an interesting pattern of the distribution of risk factors around cases and controls. Preventive categories were tightly clustered around controls, whereas risk categories were more diffuse around cases. These results suggest that, control herds were doing most practices correctly. The results also imply that case herds did not necessarily do all practices wrong, however, failing to follow a comprehensive control system may cause the herd to be a case.

8.4 Identification and characterization of lipolytic and proteolytic bacteria in pasteurized milk.

In this study, BTM from 100 farms was subjected to laboratory pasteurization (62.8 °C for 30 min). The lipolytic and proteolytic activity of the surviving bacteria was determined under conditions that approximate poor refrigeration. The results of this study showed that the predominant isolates of pasteurized milk were Gram-positive rods (83%, mainly *Bacillus* spp.), followed by Gram-positive cocci (17%, mainly *Staphylococcus* spp.) The predominance of *Bacillus* spp. in pasteurized milk has been documented in recent studies (7, 8). Most of the isolates in this study had proteolytic or lipolytic activity or both, which indicates their potential of causing spoilage of pasteurized milk.

8.5 Mastitis associated pathogens as a source of elevated total bacterial counts in bulk tank milk

In this study, BTM samples (n=128) that had Bactoscan count >8,000 bacteria/ml, were analyzed for TAC on Petrifilm, and for streptococcus, staphylococcus, and Gram-negative bacterial counts using specific media. The results showed that most of the time (72/89), mastitis-associated pathogens were not a significant contributor to high TAC (>10,000 cfu/ml) in BTM. However, in 17 (19%) out of 89 samples, mastitis-associated pathogens had a significant proportional contribution (≥0.25) to the TAC. *Streptococcus uberis, Escherichia coli*, and coagulase negative staphylococci were the main contributors. Because environmental streptococci, CNS, and coliform could originate from the environment or from infected cows, further knowledge of individual cow cultures and prevalence of mastitis in the herd would be required to determine the source of these organisms.

8.6 Overall Conclusion

The work presented in this thesis indicates that bacterial tests used in this study (TAC, PIC, LPC, and CC) can be used to quantify the quality of raw milk. Each of these tests examines a different population of bacteria within raw milk and can be used to predict where hygiene deficits occur on the farm. Therefore, using multiple bacterial tests that estimate specific groups of bacteria in BTM is required to get a more detailed picture on hygienic practices employed on the farm during collection, handling and storage of BTM. Additionally, assessment of hygienic quality of raw milk in a herd should rely on several measurements of each test and not on a single value. In general environment and cow factors are strongly associated with TAC and PIC and equipment hygiene is most closely associated with LPC and CC.

Gram-positive rods, mainly *Bacillus* spp., are the primary organisms surviving laboratory pasteurization and represent a big challenge to the shelf-life of pasteurized milk and dairy products.

Mastitis associated pathogens can be found in extremely high counts and can be associated with elevated total bacterial count in BTM.

8.7 Strengths and limitations of this work

A strength of this study was the use of several tests at the same time for evaluation of raw milk quality, which allowed us to get more information about the relationship between these tests. Another strength was using longitudinal data, which allowed the detection of changes in test values over time. In the case-control study, evaluation of large number of risk factors by highly trained investigators, blindness of the investigators to the case-control status of the farm, and direct observation and evaluation of risk factors lead to more accurate and unbiased independent variables. A potential weakness of this

study was the use of producer completed questionnaires which may introduce some bias in risk factors classification, however this issue has been addressed by the case-control study. Another weakness is using a relatively small sample size for case-control study, in particular the part dealing with bacterial class specific risk factors. This issue, in conjunction with multicollinearity in the data prevented a full multivariable model evaluation of these data.

8.8 Future work

Although the research within this thesis does provide substantial information on risk factors for raw milk bacterial quality, there is still need for additional research to complete this work and to get a better understanding of the relationship between on-farm management practices and raw milk bacterial quality. A larger case-control study at individual test levels may be required for quantifying the effect of risk factors for individual bacterial class in multivariable models. As noted, small sample size and multicollinearity resulted in problems for fitting multivariable models. In this work, 2 methods were used for dealing with multicollinearity, the use of Cronbach's alpha for grouping predictors measuring the same management procedures and the use of multiple correspondence analyses to summarize graphically the relationship between different predictors and the outcome variables. The results of this work showed that TAC and PIC were moderately correlated. Additionally, risk factors for both TAC and PIC were very similar. This raises a question of whether there is additional value in doing PIC after TAC is done. More research to determine the unique attributes of high PIC herds is required. The predominance of *Bacillus* spp. in pasteurized milk and the lipolytic and proteolytic activity shown by these bacteria under refrigeration condition represent a big challenge to

the shelf-life of pasteurized milk. Further investigations of on-farm risk factors for contamination of raw milk with *Bacillus* spp. is required for effective control of these spoilage bacteria. The isolation of *S. aureus* from pasteurized milk requires further investigation of thermotolerance of this organism under both laboratory and commercial pasteurization. This study uses a case control methodology to determine associations between risk factors and milk quality. Stronger causal links between control measures (based on the risk factors identified) could be better established using a clinical trial model.

8.9 References

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APPENDICES

Appendix A: Mail out survey

The dairy research group, at the Atlantic Veterinary College, is working closely with the dairy industry (producers and processors) to improve milk quality in the province. Thank you for taking the time to complete this survey, it should take **less than 15 minutes**. This survey is essential to our understanding of the factors that promote high quality raw milk on farms.

There are specific questions about cleaners and sanitizers. As a result, it may be most efficient to answer **in the milkhouse**, where this information is available.

All producers who complete the survey will have their names entered for a draw for $100 in ADL products.

Date survey completed: .....

Farmname: .....

Contact person: .....

Telephone: (.....) ..... (home)

(.....) ..... (cell phone)

Milk Shipping number: .....

ADLIC Number .....

I grant permission for Dr. Greg Keefe to access my ADLIC data for research purposes

Signature .....

All individual farm information will be kept **confidential**.
Thank you for completing the survey! Please return in the enclosed self-addressed envelop.

For any questions and inquiries please contact:
Dr. Greg Keefe
Professor, Dairy Health Management
Dept. Health Management
Atlantic Veterinary College
tel: 566 0968
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1. General questions about the farm

1.1. How many cows do you currently have?

(a) ..... lactating cows

(b) ..... dry cows

(c) .....bred heifers

1.2 Type of housing for the lactating cows, dry cows and bred heifers (☒ **all** that apply):

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Tie-stall*	☐ *Tie-stall*	☐ *Tie-stall*
☐ *Free-stall*	☐ *Free-stall*	☐ *Free-stall*
☐ *Manure / straw pack*	☐ *Manure / straw pack*	☐ *Manure / straw pack*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

1.3 Type of milking system

☐ Tie-stall with bucket milkers
☐ Tie stall with pipeline
☐ Flat parlor
☐ Herring bone parlor
☐ Parallel *(Side by side)* parlor
☐ Tandem *(Side-opening)* parlor
☐ *Other: (please specify)* .....

1.4 (a) Do the **lactating cows** go outside in the Summer?

☐ *No, they stay in the barns all year round*
☐ Yes, *but they only have access to an exercise yard (less than 5 acres / 100 cows)*
☐ *Yes, they go on pasture from the month ..... until .....*

2. Cow Cleanliness and hygiene

2.1 What material does the stall base consist of? (☒ all that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Concrete*	☐ *Concrete*	☐ *Concrete*
☐ *Mattress*	☐ *Mattress*	☐ *Mattress*
☐ *Rubber mat*	☐ *Rubber mat*	☐ *Rubber mat*
☐ *Clay*	☐ *Clay*	☐ *Clay*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.2 What material do you use as bedding? (☒ all that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *None*	☐ *None*	☐ *None*
☐ *Sawdust*	☐ *Sawdust*	☐ *Sawdust*
☐ *Shavings*	☐ *Shavings*	☐ *Shavings*
☐ *Sand*	☐ *Sand*	☐ *Sand*
☐ *Straw*	☐ *Straw*	☐ *Straw*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.3 How often do you **clean out the manure** in the stalls? (for example scraping the back 1/2 of the stalls out) (☒ **all** that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Twice a day or more*	☐ *Twice a day or more*	☐ *Twice a day or more*
☐ *Once a day*	☐ *Once a day*	☐ *Once a day*
☐ *Once every two days*	☐ *Once every two days*	☐ *Once every two days*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.4 How often do you **change the bedding** in the stalls (☒ **all** that apply)?

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Once a day*	☐ *Once a day*	☐ *Once a day*
☐ *Once every two days*	☐ *Once every two days*	☐ *Once every two days*
☐ *Twice a week*	☐ *Twice a week*	☐ *Twice a week*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.5 If you have a free-stall *(Tie-stall herds go to question 2.6)*
(a) How are the alleys cleaned (☒ **all** that apply)?

☐ *Manual*
☐ *Automated*
☐ *Skid-steer or tractor*
☐ *Other: (please specify)* .....

(b) How often are the alleys scraped per day?

..... *times / day*

2.6 Do you clip or flame udders?

☐ *No*
☐ *Clip,* ..... *times / year*
☐ *Flame,* ... . *times / year*

2.7 Do you clip or dock tails?

☐ *No*
☐ *Clip, ... times / year*
☐ *Dock*

2.8 (a) Do you have a **maternity pen / calving stall**?

☐ *Yes* ☐ *No (please proceed to question 2.9)*

(b) What kind of bedding material do you use in the maternity pen?

☐ *None*
☐ *Sawdust*
☐ *Shavings*
☐ *Sand*
☐ *Straw*
☐ *Other: (please specify)* .....

(c) How often is the bedding replaced by clean bedding?

☐ *After every calving*
☐ *Other: (please specify)* .....

2.9 What percentage of cows have visible mud or manure on teats prior to udder preparation in the summer?

Never occurs <5% 5-10% 10-20% 20-30% >30%

2.10 What percentage of cows have visible mud or manure on teats prior to udder preparation in the winter?

Never occurs <5% 5-10% 10-20% 20-30% >30%

3. Milking procedures and mastitis control

3.1 Mark ☒ the appropriate boxes (1 per section) to complete the following table describing your pre-milking practices

	1. No preparation prior to unit attachment
If you do some preparation, select the most correct from each section below	
	2a. Dry Wipe all teats
	2b. Dry Wipe dirty teats only
	2c. Do not Dry Wipe
	3a. Wash all teats with water with disinfectant
	3b. Wash dirty teats only with water with disinfectant
	3c. Wash all teats with water (without disinfectant)
	3d. Wash dirty teats only with water (without disinfectant)

	3e. Do not use water to clean teats
	4a. Apply pre-dip using a teat dipper
	4b. Apply pre-dip using a teat sprayer
	4c. Use commercial wet disinfectant towel (such as Readywipe®)
	4d. Do not use pre-dip or disinfectant towel
	5a. Strip foremilk from all cows
	5b. Strip foremilk from some selected cows
	5c. No foremilk stripping
	6a. Use separate paper towel/newspaper for each cow when cleaning/drying udder
	6b. Use same paper towel/newspaper for more than 1 cow when cleaning/drying udder
	6c. Use separate cloth for each cow when cleaning/drying udder
	6d. Use same cloth for more than one cow when cleaning/drying udder
	6e. Do not use any paper towel/newspaper or clothes
	Other – additional pre-milking procedures not covered by above methods

3.2 Mark ☒ the appropriate boxes to complete the following table, describing your milking and post- milking practices. Select the most correct from each section below.

	1a. All milkers wear latex or similar gloves during milking
	1b. Some milkers wear latex or similar gloves during milking
	1c. Milkers do not wear latex or similar gloves during milking
	2a. Post milking teat dip is applied after milking using a dipper
	2b. Post milking teat dip is applied after milking using a sprayer
	2c. Post milking teat dip is not used

3.3. How many cows have mastitis (as defined in each line below) on your farm in a typical period?

_____ mild cases with abnormal milk (with or without swelling in the quarter) per (month/year) *circle one*

_____ more severe cases; abnormal milk **AND** the cow is sick (not eating) per (month/year) *circle one*

3.4 Do you milk cows with **mastitis** last and/or with a separate unit?
☐ *Yes* ☐ *No*

3.5 Do you milk cows with **high somatic cell count** cows last and/or with a separate unit?
☐ *Yes* ☐ *No*

3.6 What proportion of cows are dry-cow treated with antibiotics at the end of lactation? *Approximately* ..... %

3.7 (a) Do you use *Orbeseal®* at dry off?

☐ *Yes* ☐ *No (please proceed to question 4.1)*

(b) What proportion of cows are dry-cow treated with *Orbeseal®*?

Approximately ..... %

(c) Do you use *Orbeseal®* in combination with antibiotics?

☐ *Yes, always in combination with antibiotics*
☐ *Sometimes*
☐ *No, I always use Orbeseal® alone*

4. Equipment maintenance and cleaning

4.1 How frequently are regular milking system maintenance checks by these individuals? (☒ one per section)

(a) Self

Daily Weekly Monthly Quarterly Annually Never

(b) Dealer

Monthly Quarterly Twice a year Annually Once per 2 years Never

(c) Udder Health Technician

Monthly Quarterly Twice a year Annually Once per 2 years Never

(d) Other (specify)

Daily Weekly Monthly Quarterly Annually Never

4.2 How do you clean the milk contact surface (inside) of your **milking equipment**?

Automatic system Manual Other _____

4.3 List the cleaners and sanitizers used in the **milking system** and frequency of use.

a_____ Twice a day Daily Other*(specify)*_____

b_____ Twice a day Daily Other*(specify)*_____

c_____ Twice a day Daily Other*(specify)*_____

d_____ Twice a day Daily Other*(specify)*_____

4.4 How do you routinely clean the milk contact surface (inside) of your **bulk tank**?

Automatic system Manual Other _____

4.5 If you have an automated system, do you additionally manually clean the inside of your bulk tank?

☐ No ☐ Yes (estimate frequency) _____ times per week/month/year (circle)

4.6 List the cleaners and sanitizers used in the **bulk tank** and frequency of use.

a_____ After each pickup Other *(specify)* _____

b_____ After each pickup Other *(specify)* _____

c_____ After each pickup Other *(specify)* _____

d_____ After each pickup Other *(specify)* _____

4.7 When was the most recent analysis of your equipment wash cycle (water temperatures, chemical analysis, slugging action etc)?

Within the last year (approximate date _____)
1-2 years ago Longer than two years ago
I do not recall ever having a wash cycle analysis

4.8 How long does it take, after the first milking of the pickup cycle, for the bulk tank to cool to the required temperature (40°F or 4°C) (ie until compressor shuts off)?

Within 15 min 15-30 min 30-60 min 60-90 min
Other (please specify) Do not know

4.9 (a) How often do you check your milk house water supply for bacterial contamination?

More than once/year Once/year Every second year
Longer than every second year Never Other (please specify)

(b) If you had your water checked for bacteria, were any problems identified on the the most recent test? ☐ Yes ☐ No

4.10 Do you have a water purification system (UV light or similar) for your wash water?
☐ Yes ☐ No

4.11 (a) How often do you check your milk house water supply for hardness?

More than once/year Once/year Every second year
Longer than every second year Never Other (please specify)

(b) If you had your water checked for hardness were any problems identified on the most recent test? ☐ Yes ☐ No

4.12 Do you have a water softener for your wash water?
☐ Yes ☐ No

4.13 Do you have a plate cooler, free heater or other device for pre-cooling milk prior to entry into the bulk tank? ☐ Yes ☐ No

Appendix B: On-farm survey

Date survey completed: ..... Milk Shipping number: .....

Farmname: ..... Contact person:
.....

1. General questions about the farm

1.1. How many cows do you currently have?

(a) ..... lactating cows (b) ..... dry cows (c) .....bred heifers

1.2 Type of housing for the lactating cows, dry cows and bred heifers (☒ **all** that apply):

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Tie-stall*	☐ *Tie-stall*	☐ *Tie-stall*
☐ *Free-stall*	☐ *Free-stall*	☐ *Free-stall*
☐ *Manure / straw pack*	☐ *Manure / straw pack*	☐ *Manure / straw pack*
☐ *Other: (please specify)*	☐ *Other (please specify)*	☐ *Other: (please specify)*
.....	.....	
.....		

1.3 Type of milking system

☐ Tie-stall with bucket milkers
☐ Tie stall with pipeline
☐ Flat parlor
☐ Herring bone parlor
☐ Parallel *(Side by side)* parlor
☐ Tandem *(Side-opening)* parlor
☐ *Other: (please specify)* .....

1.4 (a) Do the **lactating cows** go outside in the Summer?

☐ *No, they stay in the barns all year round*
☐ Yes, *but they only have access to an exercise yard (less than 5 acres / 100 cows)*
☐ *Yes, they go on pasture from the month ..... until .....*

2. Cow Cleanliness and hygiene

2.1 What material does the stall base consist of? (☒ all that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Concrete*	☐ *Concrete*	☐ *Concrete*
☐ *Mattress*	☐ *Mattress*	☐ *Mattress*
☐ *Rubber mat*	☐ *Rubber mat*	☐ *Rubber mat*
☐ *Clay*	☐ *Clay*	☐ *Clay*
☐ Gravel	☐ Gravel	☐ Gravel
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.2 What material do you use as bedding? (☒ all that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *None*	☐ *None*	☐ *None*
☐ *Sawdust*	☐ *Sawdust*	☐ *Sawdust*
☐ *Shavings*	☐ *Shavings*	☐ *Shavings*
☐ *Sand*	☐ *Sand*	☐ *Sand*
☐ *Straw*	☐ *Straw*	☐ *Straw*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.2.1 If Sawdust or shavings are used are they Kiln Dried? Y N

2.3 How often do you **clean out the manure** in the stalls? (for example scraping the back 1/2 of the stalls out) (☒ **all** that apply)

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Twice a day or more*	☐ *Twice a day or more*	☐ *Twice a day or more*
☐ *Once a day*	☐ *Once a day*	☐ *Once a day*
☐ *Once every two days*	☐ *Once every two days*	☐ *Once every two days*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

....
.....

2.4 How often do you **change the bedding** in the stalls (☒ **all** that apply)?

(a) **Lactating cows:**	(b) **Dry cows:**	(c) **Bred heifers:**
☐ *Twice a day or more*	☐ *Twice a day or more*	☐ *Twice a day or more*
☐ *Once a day*	☐ *Once a day*	☐ *Once a day*
☐ *Once every two days*	☐ *Once every two days*	☐ *Once every two days*
☐ *Twice a week*	☐ *Twice a week*	☐ *Twice a week*
☐ *Once a week*	☐ *Once a week*	☐ *Once a week*
☐ *Other: (please specify)*	☐ *Other: (please specify)*	☐ *Other: (please specify)*

.....

2.5 If you have a free-stall *(Tie-stall herds go to question 2.6)*

(a) How are the alleys cleaned (☒ **all** that apply)?

- ☐ *Manual*
- ☐ *Automated*
- ☐ *Skid-steer or tractor*
- ☐ *Other: (please specify)* .....

(b) How often are the alleys scraped per day?

..... *times / day*

2.6 Do you clip or flame udders?

☐ *No*
☐ *Clip,* ..... *times / year*
☐ *Flame,* ... . *times / year*

2.7 Do you clip or dock tails?

☐ *No*
☐ *Clip,* ..... *times / year*
☐ *Dock*

2.8 (a) Do you have a **maternity pen / calving stall**?

☐ *No (please proceed to question 2.9)* ☐ *Yes* How many Maternity pens _____

(b) What kind of bedding material do you use in the maternity pen?

☐ *None*
☐ *Sawdust*
☐ *Shavings*
☐ *Sand*
☐ *Straw*
☐ *Other: (please specify)* .....

(c) How often is the bedding replaced by clean bedding in the maternity pen?

☐ *Once a day* ☐ *Once every two days* ☐ *Twice a week*
☐ *Once a week* ☐ *After every calving* ☐ *Other: (please specify)*

2.9 What percentage of cows have visible mud or manure on teats prior to udder preparation in the summer?

Never occurs <5% 5-10% 10-20% 20-30% >30%

2.10 What percentage of cows have visible mud or manure on teats prior to udder preparation in the winter?

Never occurs <5% 5-10% 10-20% 20-30% >30%

3. Milking procedures and mastitis control

3.1 Mark ☒ the appropriate boxes (1 per section) to complete the following table describing your pre-milking practices

	1. No preparation prior to unit attachment		
If you do some preparation, select the most correct from each section below		**Order**	**Product**
	2a. Strip foremilk from all cows		
	2b. Strip foremilk from some selected cows		
	2c. No foremilk stripping		
	3a. Dry Wipe all teats		
	3b. Dry Wipe dirty teats only		
	3c. Do not Dry Wipe		
	4a. Wash all teats with water with udder wash		

	4b. Wash dirty teats only with water with udder wash		
	4c. Wash all teats with water (without udder wash)		
	4d. Wash dirty teats only with water (without udder wash)		
	4e. Do not use water to clean teats		
	5a. Apply pre-dip using a teat dipper		
	5b. Apply pre-dip using a teat sprayer		
	5c. Use commercial wet disinfectant towel (such as Readywipe®)		
	5d. Do not use pre-dip or disinfectant towel		
	6a. Use separate paper towel/newspaper when drying udder		
	6b. Use same paper towel/newspaper for more than 1 cow		
	6c. Use separate cloth when cleaning/drying udder		
	6d. Use same cloth for more than one cow		
	6e. Do not use any paper towel/newspaper or clothes		
	Other – additional pre-milking procedures not covered by above methods		

3.2 Mark ☒ the appropriate boxes to complete the following table, describing your milking and post-milking practices. Select the most correct from each section below.

	1a. All milkers wear latex or similar gloves during milking	
	1b. Some milkers wear latex or similar gloves during milking	
	1c. Milkers do not wear latex or similar gloves during milking	
	2a. Post milking teat dip is applied after milking using a dipper	Brandname:
	2b. Post milking teat dip is applied after milking using a sprayer	
	2c. Post milking teat dip is not used	

3.3. How many cows have mastitis (as defined in each line below) on your farm in a typical period?

_____ mild cases with abnormal milk (with or without swelling in the quarter) per (month/year) *circle one*

_____ more severe cases; abnormal milk **AND** the cow is sick (not eating) per (month/year) *circle one*

3.4 Do you milk cows with **mastitis** last and/or with a separate unit?

☐ *Yes* ☐ *No*

3.5.1 Do you milk cows with **high somatic cell count** cows last and/or with a separate unit in the summer?

☐ *Yes* ☐ *No*

3.5.2 Do you milk cows with **high somatic cell count** cows last and/or with a separate unit in the winter?

☐ *Yes* ☐ *No*

3.6 What proportion of cows are dry-cow treated with antibiotics at the end of lactation?

Approximately ..... %

3.7 (a) Do you use *Orbeseal*® at dry off?

☐ *Yes* ☐ *No (please proceed to question 4.1)*

(b) What proportion of cows are dry-cow treated with *Orbeseal®*?

Approximately ..... %

(c) Do you use *Orbeseal®* in combination with antibiotics?

☐ *Yes, always in combination with antibiotics*
☐ *Sometimes*
☐ *No, I always use Orbeseal® alone*

4. Equipment maintenance and cleaning

4.1 How frequently are regular milking system maintenance checks by these individuals? (☒ one per section)

(a) Self

Daily Weekly Monthly Quarterly Annually
Never Other specify _____

(b) Dealer

Monthly Quarterly Twice a year Annually Once per 2 years
Never Other specify _____

(c) Udder Health Technician

Monthly Quarterly Twice a year Annually Once per 2 years
Never Other specify _____

(d) Other people (specify who)

Daily Weekly Monthly Quarterly Annually Never
Other specify _____

4.2 How do you clean the milk contact surface (inside) of your **milking equipment**?

Automatic system Manual Other _____

4.3 List the cleaners and sanitizers used in the **milking system** and frequency of use.

a_____ Twice a day Daily Other*(specify)*_____

b_____ Twice a day Daily Other*(specify)*_____

c_____ Twice a day Daily Other*(specify)*_____

d_____ Twice a day Daily Other *specify)*_____

4.4 How do you routinely clean the milk contact surface (inside) of your **bulk tank**?

Automatic system Manual Other _____

4.5 If you have an automated system, do you additionally manually clean the inside of your bulk tank?

☐ No ☐ Yes (estimate frequency) _____ times per week/month/year

4.6 List the cleaners and sanitizers used in the **bulk tank** and frequency of use.

a_____ After each pickup Other *(specify)* _____

b_____ After each pickup Other *(specify)* _____

c_____ After each pickup Other *(specify)* _____

d_____ After each pickup Other *(specify)* _____

4.7 a) Do you have a protocol to insure that chemicals for cleaning do not run out (fill reserviors)

□ No □ Yes (estimate frequency of refill) _____ times per week/month/year

b) How many times have your chemical reservoirs run out in the last 3 months

Never Once Twice More than 2 times

4.8 . When was the most recent analysis of your equipment wash cycle (water temperatures, chemical analysis, slugging action etc)?

Within the last year (approximate date _____)
1-2 years ago Longer than two years ago
I do not recall ever having a wash cycle analysis

4.9 How long does it take, after the first milking of the pickup cycle, for the bulk tank to cool to the required temperature (40°F or 4°C) (ie until compressor shuts off)?

Within 15 min 15-30 min 30-60 min 60-90 min
Other (please specify) Do not know

4.10 Do you have a device to improve cooling times/energy use in the bulk tank? (circle all that apply)

Plate cooler Free heater Other device *(specify)* _____

5. Water

5.1 What is your water source

Deep well (>100 feet) Shallow well (<100 feet) Spring
Pond Municipal Other (please specify)

5.2 (a) How often do you check your milk house water supply for bacterial contamination?

More than once/year Once/year Every second year
Longer than every second year Never Other (please specify)

(b) If you had your water checked for bacteria, were any problems identified on the the most recent test? □ Yes □ No

(c) If yes, describe the corrective action taken _____

5.3 (a) How often do you check your milk house water supply for chemical composition (Hardness, Minerals, pH)?

More than once/year Once/year Every second year
Longer than every second year Never Other (please specify)

(b) If you had a chemistry analysis on your water, were any problems identified on the the most recent test? □ Yes □ No

(c) If yes, describe the corrective action taken _____

5.4 Do you have a water purification system (UV light or similar) for your wash water?
□ Yes □ No

5.5 Do you have a water softener for your wash water?
□ Yes □ No

Appendix C: On-farm data collection: Equipment wash analysis

Date of visit: .....

Farmname: ..... Contact person: .....

Milk Shipping number: .....

Section 1. Equipment Cleaning

A. Wash Analysis Bulk Tank

i Temperature and Chemical Analysis

Cycle	Fill Temp	Drain Temp	pH	Alkalinity	Chlorine	Comments
Pre Rinse						
Rinse						
Pre Wash						
Alkaline Wash						
Post Wash Rinse						
Acid Rinse						

ii. Overall Water Hardness Score: _____

iii. Cleaning ball function:

Poor				Excellent
1	2	3	4	5

D. Wash Analysis Pipeline

Cycle	Start Temp	End Temp	pH	Alkalinity	Chlorine	Comments
Rinse						
Alkaline Wash						
Acid Wash						
Sanitizer						

E. Pipeline Slugging Action

Slugging Action		
Beginning	**Mid-Line**	**End**
Poor Good **1 2 3 4 5**	**Poor Good** **1 2 3 4 5**	**Poor Good** **1 2 3 4 5**

Air Injector

i Open (Firing time) _____
ii Closed time _____
iii Approx air volume _____

Other Observations on System		Poor				Good
i	Bypass through wash diversion valve	1	2	3	4	5
ii	Too much water in the sink	1	2	3	4	5
iii	Air entering the units or fill pipe at sink	1	2	3	4	5
iv	Slope	1	2	3	4	5
v	Trapping out	1	2	3	4	5

Other Comments

Section 2. Milking practices

A. Observe a minimum of 2 shifts of the milk equipment and record the udder preparation methods on the following chart:

	1. No preparation prior to unit attachment	
	If prep is done, describe and give order completed	**Order**
	2a. Strip foremilk from all cows	
	2b. Strip foremilk from some selected cows	
	2c. No foremilk stripping	
	3a. Dry Wipe	
	3b. Do not Dry Wipe	
	4a. Wash teats with water with commercial udder wash	
	4b. Wash all teats with water (without udder wash)	
	4c. Do not use water to clean teats	
	5a. Apply pre-dip using a teat dipper	
	5b. Apply pre-dip using a teat sprayer	
	5c. Use commercial wet disinfectant towel (such as Readywipe®)	
	5d. Do not use pre-dip or disinfectant towel	
What is the contact time for disinfectant before wipe off? <15s 15-30s 30-60s 1-2m >2 m		
	6a. Use separate paper towel/newspaper for each cow when cleaning/drying udder	
	6b. Use same paper towel/newspaper for more than 1 cow when cleaning/drying udder	
	6c. Use separate cloth for each cow when cleaning/drying udder	
	6d. Use same cloth for more than one cow when cleaning/drying udder	
	6e. Do not use any paper towel/newspaper or clothes	
	Other – additional pre-milking procedures not covered by above methods	

B. Milking and Post-Milking

	1a. Milkers wear latex or similar gloves during milking
	1b. Some milkers wear latex or similar gloves during milking
	1c. Milkers do not wear latex or similar gloves during milking
	2a. Post milking teat dip is applied after milking using a dipper
	2b. Post milking teat dip is applied after milking using a sprayer
	2c. Post milking teat dip is not used

C. Record squawks and fall offs

i ___ squawks per ___ cow milkings observed

ii ___ unit fall offs per ____ cow milkings observed

D. Score teat preparation and teat ends using the charts provided.
1= clean **2**= teat dip residue **3**= some feces/dirt **4**= excess dirt/feces

	Teat Cleanliness			
Cow	RF	LF	RR	LR
1	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
2	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
3	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4

	Teat End Score (use chart)			
Cow	RF	LF	RR	LR
1	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
2	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
3	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4

Appendix D: On-farm data collection: Environment, cow, and equipment hygiene

Date of visit: .....

Farmname: .....

Contact person: .....

Milk Shipping number: .....

Has a mailout survey been completed for this farm ☐

Section 1: Environmental Scoring

1. By formal random system (attached) select **4-6** milking cow stalls, **2** dry cow stalls and **1** calving pen

2. Score stall cleanliness and sample for bedding count and dry matter (12-15 small bites per sample in back ½ of stall)

	Score				√ bedding sample	Stall Photo #
Milking stall 1	1	2	3	4		
Milking stall 2	1	2	3	4		
Milking stall 3	1	2	3	4		
Milking stall 4	1	2	3	4		
Milking stall 5	1	2	3	4		
Milking stall 6	1	2	3	4		
Dry cow stall 1	1	2	3	4		
Dry cow stall 2	1	2	3	4		
Calving pen 1	1	2	3	4		

Section 2. Cow hygiene

Score cows in the above selected stalls using chart provided – If dry cows are loose housed score 2 random cows

	Udder	Lower legs	Flank/thigh	Cow Photo #
Milking cow 1	1 2 3 4	1 2 3 4	1 2 3 4	
Milking cow 2	1 2 3 4	1 2 3 4	1 2 3 4	
Milking cow 3	1 2 3 4	1 2 3 4	1 2 3 4	
Milking cow 4	1 2 3 4	1 2 3 4	1 2 3 4	
Milking cow 5	1 2 3 4	1 2 3 4	1 2 3 4	
Milking cow 6	1 2 3 4	1 2 3 4	1 2 3 4	
Dry cow 1	1 2 3 4	1 2 3 4	1 2 3 4	
Dry cow 2	1 2 3 4	1 2 3 4	1 2 3 4	
Calving 1	1 2 3 4	1 2 3 4	1 2 3 4	

Section 3. Equipment Cleaning

A. Record the presence and nature of any film in the Bulk tank when empty and dry using 1 million foot candle light (circle appropriate level)

i Clean and shiney

ii. Film covering:

< 1% (patch) 1-5% 5-15% 15-40% >40%

iii Describe film: (circle)

White Blue Other_____

iv Describe location_____

B. Biofilm assessment (Using Ecolabs Pocketswabs)

Bulk tank		Milking System	
Site	**Score**	**Site**	**Score**
Agitator		Inlet 1* far	
Base back wall		Inlet 2* near	
Outlet		Receiver jar	
		Diverter valve	
		Crossover pipe (sock)	

* one on each side of barn one far and one near to milkhouse

C. Visual score of pipeline (at junction)

i Clean and shiney

ii. Film covering:

< 1% (patch) 1-5% 5-15% 15-40% >40%

iii Describe film: (circle)

White Blue Other_____

iv Describe location_____

Section 4. Cooling system and milk filter

A. Activate and place the data logger in the bulk tank ☐

B. Place cooler with baggies for subsequent 4 filters ☐

D. Place sign for trucker to retreive data logger and filter cooler ☐

Section 6. Other

B. After visit procedures

Submit sample for bedding dry matter ☐

Submit sample for bedding count ☐